IN VITRO GERMINATION OF *CYPRIPEDIUM* SPECIES

by

Mary A. De Pauw

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MARY A. DEPAUW

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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ABSTRACT

De Pauw, Mary A. M.Sc., Department of Plant Science, University of Manitoba. *In Vitro* Germination of *Cypripedium* Species. Major Professor: Dr. W.R. Remphrey.

The *in vitro* germination of *Cypripedium candidum* Muhl. ex Willd., *C. calceolus* L. var. *parviflorum* (Salisb.) Fern. and *C. reginae* Walt. was investigated. These species are becoming rare, especially *C. candidum*, making an efficient method of propagation desirable. There is also interest in the nursery trade regarding their potential as herbaceous perennials because of their beautiful flowers.

Little is known about the specific germination requirements of *Cypripedium* species. To improve *in vitro* germination, three media and different times of seed collection were compared. Seed pods were collected at regular intervals after pollination and seed inoculated onto three media, Harvais, Van Waes and Debergh, and modified Norstog. Germination was then assessed at 4-week intervals for 20 weeks. Except for *C. candidum* in 1989, where germination peaked for seed collected 6 weeks after pollination, seed collected eight weeks after pollination gave the highest germination. There were differences among media, but their effects on germination were not consistent and depended on the year, date of collection and the species. Subsequent development of protocorms was superior in all cases on modified Norstog medium. The effect of cold treatment of seed on the germination of *C. candidum* was investigated. The results were highly variable and thus its importance in improving germination is questionable and warrants further investigation.

Using the information obtained in the first two years, subsequent experiments were performed using modified Norstog medium and seed collected eight weeks after pollination to investigate the requirement for cytokinins and the cytokinin preference for *in vitro* germination and protocorm development of *C. candidum*. Two of the three cytokinins tested, BA and 2iP, increased germination at concentrations of up to 0.8 mg L^{-1} compared to a control without cytokinins. Kinetin, on the other hand, had little effect regardless of concentration. Nine protocorm morphological types were identified. The morphological types observed with kinetin were similar to those seen in the control, whereas on BA and 2iP different morphological types were observed. Protocorms developed faster on all three cytokinin treatments compared to the control. Benzyl adenine delayed root development at the highest concentrations. A model is proposed to explain the effect of cytokinins on protocorm development.

FOREWORD

This thesis has been written in manuscript style. Chapter 1, entitled "*In vitro* germination of three *Cypripedium* species in relation to time of seed collection, media and cold treatment", has been accepted for publication (April 23 1993) in the Canadian Journal of Botany. Chapter 2 entitled "The cytokinin preference for germination and protocorm growth of *Cypripedium candidum* Muhl.", will be submitted for publication to the journal, Physiologia Plantarum.

INTRODUCTION

Cypripedium species, commonly called lady's-slippers, are native wildflowers belonging to the *Orchidaceae* family. They are renowned for their beautiful and showy flowers, which make them desirable for commercial production. Unfortunately, attempts to domesticate these plants have usually failed. They grow slowly and become dormant easily, thus making vegetative propagation impractical. Standard seed germination techniques are also not practical because very specific conditions are required, including a symbiotic relationship with endophytic fungi. A reliable and reproducible propagation method is required for commercial production. Tissue culture may provide a solution, and *in vitro* seed germination of *Cypripedium* species has great potential because it is a nondestructive propagation method and seeds are produced in great abundance.

Another important reason for developing viable propagation methods is the problem of declining populations. *Cypripedium* species, as the case with many native wildflowers, are threatened because of habitat destruction caused by both human activities and natural processes. This is especially true for *C. candidum* Muhl. which is listed as endangered in all of Canada, including the Province of Manitoba. Another concern is the removal of these plants from their native habitats for resale in nurseries and farmers' markets, and for illegal trade outside of the country, especially in the case of *C. calceolus* L. Reliable propagation methods would eliminate the need to remove these plants from their native habitats and reestablishment in the wild would help save those species in danger of extinction.

Research on the *in vitro* germination of *Cypripedium* species has been limited and the results often contradictory. Much of the literature is also only observational in nature. Germination is often poor and slow in these orchids and little is known about their specific germination requirements, especially for *C. candidum*. The objectives of this research are to improve *in vitro* germination of *Cypripedium* species and to learn more about their germination requirements.

In general, mature seed of *Cypripedium* species germinates very poorly, with germination percentages often as low as, or lower than, 1%. Use of immature seed taken from green pods improves germination, but the exact time of harvest to optimize germination is not known. Seed dormancy has been suggested as one cause of poor germination. Research results on the use of cold treatment to break dormancy are contradictory. The kinds of media used to germinate native orchid seeds that has been reported in the literature is vast and their components vary dramatically. Using three *Cypripedium* species, *C. reginae*, *C. calceolus* var. *parviflorum*, and *C. candidum*, the initial objectives of this research are (1) to find the best time to collect seed in order to optimize germination for each species, (2) to compare the effects of three media on the germination of each species and (3) to study the effect of cold treatment on germination. Using this information, the final objective is to investigate the cytokinin requirement of *C. candidum* for germination and protocorm development.

LITERATURE REVIEW

Biology and Ecology

The genus *Cypripedium* belongs to the *Orchidaceae* family, one of the largest families of the Angiosperms. There are approximately 30 species in this genus, 11 of which occur in North America (Luer 1975). They are commonly called "lady's-slippers" because of the slipper-like appearance of the flower. The showy, usually colorful flower has one petal that is inflated to form a sac-like pouch called the lip or labellum. The other two lateral petals are flat or twisted, and often differ in color from the labellum. The dorsal sepal, similar in color to the lateral petals, extends above the pouch. The two lateral sepals are united behind the pouch, and this united structure is referred to as the synsepal (Luer 1975). The stigma, style, anthers and filaments are fused to form a single structure called a column. There are 2 functional stamens, one on each side of the column. The anthers produce masses of pollen, sometimes called pollinia, which are granular and viscid. A third sterile stamen, or staminode, forms a flat appendage at the front of the column. Flowers are usually borne singly, although some species may have as many as 12 flowers per stem (Luer 1975).

Cypripedium species are deciduous perennial terrestrial orchids with underground rhizomes which often branch, resulting in the formation of clumps of plants. The degree of branching of the rhizome depends upon the species. The leafy stem, with plicate leaves, emerges from the rhizome each spring. Flower buds are initiated in late summer of the year preceding anthesis (Curtis 1954).

Cypripedium reginae Walt., commonly called the showy lady's-slipper, is one of the largest and most beautiful of the native orchids. It can stand up to 90 cm tall, with large leaves and a large flower with a pink labellum and white petals and sepals (Luer 1975). It commonly produces two flowers per stem and occasionally even three or four. It usually grows in a mixed deciduous coniferous forest where moisture is readily available or in close proximity to a bog. In Manitoba, it flowers from late June through mid-July.

Cypripedium calceolus L. var. *parviflorum* (Salisb.) Fern. is commonly called the small yellow lady's-slipper (Luer 1975). This variety grows up to 35 cm tall and has a small flower with a yellow pouch, often with reddish-brown to purple spots, and brown to greenish sepals and petals, with varying degrees of twisting. It flowers from mid-May to late June in Manitoba. It is generally a moist woodland species, but will grow in a wide variety of habitats, including open fields in full sunlight. Luer (1975) distinguishes this plant from *C. calceolus* L. var. *pubescens* (Willd.) Correll, or the large yellow lady's-slipper, which, as the name implies, is larger in stature and has a larger flower. However, there is a high degree of variability in plant form between these two varieties and intermediates are common. This has led to some debate regarding their classification and these two plants have also been classified as separate species (Atwood 1985). In Europe, only a single form exists, resembling the large yellow lady's-slipper most closely.

Cypripedium candidum Muhl., commonly called the small white lady's-slipper, is a small dainty plant. It grows 20 to 36 cm tall, typically in compact clumps, and has

a small solitary flower per stem with a glossy white pouch, often with purple spots or veins, and greenish to brown slightly twisted petals and sepals. It flowers from mid-May to mid-June in Manitoba. It is a characteristic species of tall-grass prairie and prefers moist calcareous sites (Brownell 1981). *Cypripedium candidum* is considered to be one of the rarest lady's-slipper species in North America and in danger of extinction. Destruction of its native habitat by primarily human encroachment (mainly agricultural) as well as natural processes are considered to be the main causes of its decline (Brownell 1981). It is intolerant of shade and will often disappear when the prairie becomes invaded by woody plants. It is currently found in two Canadian provinces, Manitoba and Ontario, and in 13 U.S. states (Bowles 1983). There are 8 known sites in Southern Manitoba and two sites in Ontario (K. Johnson, pers. comm.). It is listed as endangered on the Endangered Species Acts of both Canada and the Province of Manitoba.

Cypripedium candidum and *C. calceolus* often occur together in the same habitat and flowering of the two species frequently coincides. Hybrids between these two species are believed to exist in the natural population based on intermediate plant size and flower color (Marshall 1966; Luer 1975). Hybrids between *C. candidum* and *C. calceolus* var. *parviflorum* are called *Cypripedium* x *Andrewsii* Full. and hybrids between *C. candidum* and *C. calceolus* var. *pubescens* are called *Cypripedium* x *Favillianum* Curtis (Luer 1975). Hybridization between *C. candidum* and *C. calceolus* var. *pubescens* has been confirmed using enzyme analysis (Actor 1986; Klier et al. 1991).

Pollination Biology

Cypripedium species are obligate outbreeders due to the flower structure, and are dependent on specific insect pollinators, usually bees (Stoutamire 1967; Catling and Knerer 1980; Davis 1986). The flowers act as traps for their pollinators. The bee enters the flower by an opening at the front of the labellum. Once inside the pouch, the bee is prevented from leaving the same way by the downward projecting rim of the entrance (Stoutamire 1967). To exit, the bee is led past the stigmatic surface, where it deposits any adhering pollen, and then to two small exit openings below the anthers. As the bee forces its way out one of the exit holes, it brushes past the anthers and the sticky pollen adheres to its back. Only a bee of the correct size will be successful in effecting pollination. If it is too large, it cannot enter the opening of the labellum and too small, it will not contact the pollen as it exits.

Successful pollination is often quite low and in natural populations, the occurrence of fertile seed pods is low (Davis 1986; K. Johnson, pers. comm.). The proportion of fertile pods in *C. reginae* was reported to be as low as 12% (Harvais 1980). Fruit set is believed to be pollinator limited (Davis 1986). Flowers do produce an odor which, along with their bright colors, plays a role in attracting pollinators. However, the *Cypripedium* species, like many orchids, are said to attract pollinators by "deceit" because the flower provides no nectar or other food source (Stoutamire 1967). Bees quickly learn to avoid non-rewarding flowers, resulting in low fruit set. However, *Cypripedium* species will produce seeds if mechanically pollinated (Harvais 1980).

Ovary Development

The ovary is unilocular and consists of a large number of very small ovules arranged in three double rows. In *Cypripedium* species, the ovary exhibits two phases of growth in diameter and one in length (Duncan and Curtis 1942). At the time of pollination, the ovules are at a rudimentary stage. Pod length increases steadily in the first two weeks following pollination and then levels off. During the first increase in pod diameter, from the time of pollination until approximately 4 weeks after pollination, the ovules develop from macrospore mother cells to mature macrogametophytes, ready for fertilization. Following this first growth phase, there is almost a complete cessation in pod growth, during which time fertilization takes place. The second phase of growth in pod diameter takes place after fertilization when the embryos develop rapidly. Duncan and Curtis (1942) reported that actual fertilization does not take place until 4 to 5 weeks after pollination. Carlson (1940) also found fertilization to occur very late in *C. parviflorum*, approximately 26-33 days after pollination.

Seed Characteristics

Orchid seeds, including those of *Cypripedium* species, are among the smallest angiosperm seeds, weighing from 0.3-14 μg (Arditti 1967). However, they are produced in great abundance with one pod containing from 1,300 to 4,000,000 seeds. Stoutamire (1964) estimated that one pod from *C. acaule* contained 54,180 seeds. The embryo itself has no cotyledons and simply consists of 8 to 100 mostly isodiametric cells. There is no endosperm. At one end of the embryo are enlarged partially differentiated parenchyma

cells to which the suspensor, consisting of degenerate brown cells, is attached. Lipid droplets are abundant and these are the major food reserves (Arditti 1967; Carlson 1940). Protein bodies are also abundant. The other end of the embryo consists of smaller cells with relatively large nuclei and dense cytoplasm (Arditti 1967; Harvais 1974). The mature seed contains no starch, sugar or nitrates (Carlson 1940). Immature seeds, on the other hand, do contain appreciable amounts of starch and during maturation, this starch is probably converted into lipids (Harvais 1974; Carlson 1940). The reverse is true after germination when starch abounds (Harvais 1973).

The embryo of most northern terrestrial orchids has a thin covering or scale around it consisting of the shriveled remains of the inner integument (Carlson 1940). This covering is not present in tropical orchids. The outer integuments form a thin transparent seed coat in which the embryo is suspended. The seed coat of *Cypripedium* species is varying colors of brown. Seeds of terrestrial orchids are extremely impermeable to water compared to those of epiphytic species (Van Waes and Debergh 1986a, 1986b; Pritchard 1985). This was illustrated by Burgeff (1936) who demonstrated that seeds of tropical epiphytic orchids floated on water for 1 to 9 days, whereas seeds of terrestrial species floated for several months.

Germination/The Protocorm

Germination of orchid seeds does not follow the same pathway as occurs in most flowering plants. Germination begins with a swelling of the embryo. If the seed is viable, some metabolic activity begins after the swelling, such as protein hydrolysis and

the disappearance of lipids and the formation of starch (Hadley 1982; Manning and Van Staden 1987). The embryo continues to swell until the seed coat is ruptured. The embryo is now called a protocorm. Initially the protocorm is just a small round mass in which there is little tissue differentiation. In *Cypripedium* species, the protocorm lacks chlorophyll. As the protocorm develops, it assumes a cone shape so that a basal and an apical region are established. The apical, or meristem, region develops a bulge or a small hook. The basal portion of the protocorm develops fine absorbing hairs called rhizoids. As the protocorm develops, several long roots are formed while the shoot meristem remains relatively quiescent. Growth of the protocorm from the initial stages of germination onwards is often categorized into sequential stages of development (Arditti 1967). Some refer to these stages of protocorm development as germination (Hailes and Seaton 1989).

In their natural habitat *Cypripedium* species grow very slowly. Curtis (1943) did an extensive investigation of the germination and seedling growth of 5 *Cypripedium* species in their native habitats. By careful examination of the soil around established plants, Curtis was able to identify seedlings in almost all stages of development. Seedlings develop underground and do not produce their first aerial shoot until the third year. Depending on the species, Curtis claimed that flowering did not occur for 10-16 years after germination.

An important requirement for germination and subsequent growth in nature is a mycorrhizal association with endophytic fungi. The majority of fungi associated with orchids are *Rhizoctonia* (Harvais 1974; Hadley 1982; Currah et al. 1987). The

protocorm becomes infected very soon after germination. Infection occurs through the suspensor cells or the epidermal hairs and the fungus occupies the basal cells of young protocorms. Because the protocorms and seedlings of most north temperate terrestrial orchids develop underground, they are completely heterotrophic. At this stage the fungus provides nutrients, particularly carbon compounds, and possibly specific metabolites such as enzyme precursors (Hadley and Pegg 1989).

Mature plants remain infected with the infection being restricted to the cortical tissues of the roots. This relationship appears to be much more critical for north temperate terrestrial orchids than it is for tropical, epiphytic orchids (Hadley 1982). Roots of north temperate terrestrial orchids often show complete infection of the cortex whereas tropical species show a less dense infection. Mature plants, although no longer heterotrophic, depend on the fungus during conditions of nutrient stress. Specifically, uptake of nitrogen and phosphorus is greater in mycorrhizal roots than in non-mycorrhizal roots under stress conditions (Hadley and Pegg 1989).

In Vitro Orchid Seed Germination

Because of the delicate nature of orchid seeds and the specific conditions required for germination, *in vitro* methods have been used, including both symbiotic and asymbiotic culture. Symbiotic methods involve germinating the seed on simple media along with a fungus (Clements et al. 1986; Muir 1989). In asymbiotic methods, the seeds are germinated without a fungus on a complete nutrient medium. In this case the

medium replaces the need for the fungus. Some species readily germinate asymbiotically using the appropriate medium. However, other species, especially some terrestrial species, do not germinate well under asymbiotic conditions. In this case, it has been suggested that the fungus, rather than just supplying nutrients, plays a much more important role and that the culture medium cannot completely it (Hadley 1982). Information specifically on symbiotic germination of *Cypripedium* species is very limited (Harvais 1974) and germination studies have focused on asymbiotic methods for these species. The remainder of the Literature Review will therefore focus on these asymbiotic methods.

Asymbiotic culture began when Knudson (1922) demonstrated that seeds of a tropical orchid species could be successfully germinated on a simple mineral nutrient medium with a carbohydrate source. This medium could replace the required relationship with endophytic fungi. Although Knudson's methods initially met with opposition, this medium and subsequent modifications of it are still used today for germinating the seeds of many orchid species. In fact Knudson's work marked the beginning of modern tissue culture techniques. *In vitro* propagation of tropical species using asymbiotic seed germination and meristem culture is now a widespread practice and a large-scale industry. However, north temperate terrestrial orchids have proven much more difficult to culture.

Curtis (1936) attempted to germinate 31 species of native orchid seeds, including 10 *Cypripedium* species, using Knudson medium and his own medium formulation, called Solution 5. Only 2 of the 10 *Cypripedium* species developed protocorms, referred to by

Curtis as "partial germination." Even after 2 years, "true germination," that is development of roots and shoots, was not achieved. Liddell (1944) also had negative results with seeds of *C. pubescens*, *C. acaule*, and *C. reginae* using Knudson medium.

Even recent attempts using mature seed and various media have resulted in poor germination, as low as 1% (Henrich et al. 1981; Arditti et al. 1984; Oliva and Arditti 1984). Germination is often slow, taking several months to reach the protocorm stage. It is also erratic, so several stages of protocorm development can be found within the same petri dish. Many factors have been investigated to improve asymbiotic germination.

Immature Seed

Withner (1953) reported improved germination using immature seed taken from green pods instead of mature seed and several researchers subsequently supported this finding (Stoutamire 1964; Fast 1974; Harvais 1980; Light 1989; St. Arnaud et al. 1992). There are also technical advantages to using immature seed. Sterilization of immature seed is a much simpler procedure because the entire pod can be sterilized rather than the individual seeds, which are extremely small and hydrophobic and thus are difficult to handle. Use of immature seed also decreases the waiting time until mature pods are available. However, Linden (1980) found that immature seed of *C. calceolus* did not germinate better than mature seed and Ballard (1987) reported that germination of *C. reginae* did not decrease as the seed matured. Instead germination varied randomly and unpredictably from one pod to the next.

Although the use of green pods or immature seeds is common, the time of pod collection has been inconsistent. Often the age of the seed is judged by the pod's outward appearance (e.g. green vs. brown dehisced pods) (Linden 1980; Arditti et al. 1984). This may be part of the cause of variable germination results. To alleviate this problem, hand pollination has been used to ensure seed set. Pods are then collected at specific times after pollination, for example at 6 weeks (Stoutamire 1964; Frosch 1986) and 8 to 9 weeks (Withner 1953) after pollination.

Manual pollination is a simple task to perform. The two pollinia are sticky and are easily removed from the anthers and placed on the stigmatic surface. Harvais (1980) investigated the advantages and disadvantages of mechanical cross- and self-pollination. He concluded that selfing was more productive and less laborious than crossing, and that simply using a toothpick to perform the pollination was the most effective and preferable method. He obtained up to 100% successful self-pollination. Ballard (1987) also found that selfing was as effective as cross-pollinating. He suggested that poor germination may be partly because of a lack of pollen, resulting in fewer effective fertilization events and suggested loading the flower with an excess of pollen.

Harvais (1980) recommended a different measurement for determining the time to collect seed of *C. reginae*. By using a simple iodine test for starch, he found that immature seeds, especially the integuments, have significant amounts of starch while mature seeds do not. A suitable time to collect the seed was found to be when starch has just disappeared from the integuments. Delaying seed collection past this point increased the level of dormancy factors and the risk of fungal infection (Harvais 1980).

Using a study correlating pod dimensions with internal development (Duncan and Curtis 1942), Light (1989) used pod diameter in *C. calceolus* var. *pubescens* to determine an optimum time to collect seed. She suggested harvesting pods at 40 to 55 days after pollination, which correlated to the midway point of the second phase of growth in pod diameter. At this time the embryos were 1/3 developed and consisted of approximately 9-12 cells.

Mature Seed

There have been several suggestions to explain why seeds of terrestrial orchids germinate poorly and why immature seed germinates better than mature. These factors will be discussed in detail.

Gradual Onset of Dormancy

One explanation for the poor germination of mature seed is that there is a gradual onset of dormancy as the seed matures. Mature seed therefore is highly dormant (Stoutamire 1964) and the improved germination of immature seeds may simply be due to the fact that the embryo is more metabolically awake (Withner 1953; Linden 1980) and germinates readily when taken directly from the green capsule and placed on culture medium.

Cold Treatment. Several researchers have suggested that a cold treatment may be necessary to break dormancy in mature seed (Stoutamire 1974; Fast 1982; Ballard 1987). However, results regarding the requirement for cold treatment have been very

contradictory. Ballard (1987) contends that a cold treatment is absolutely necessary for germination of *C. reginae* and suggests that dormancy, rather than gradually increasing, is established almost as soon as the capacity for germination is developed. He recommended at least 2 months of refrigeration and that "efficiency in growing cypripediums from seeds requires attention to the temperature factor."

Adequate germination and growth without the use of cold treatment has frequently been reported (Harvais 1973; Henrich 1980). Harvais (1980) found that frozen and unfrozen seeds germinated equally well and that gibberellic acid had no effect on germination. He therefore concluded that stratification is not required.

Van Waes and Debergh (1986b), using mature seed of *C. calceolus*, found that a cold treatment at 6°C for 4-8 weeks actually decreased germination (1.6±1.2%). Germination was best at a constant temperature of 23°C (35.9±4.3%). Light (1989) achieved no germination when seeds were cold-treated, regardless of the stage of seed maturity.

It should be pointed out that there is a distinction between cold treatment used to break dormancy in the seed and cold treatment used to break "epicotyl dormancy" in protocorms (Curtis 1943). Curtis (1943) indicated that leaf extension from developing protocorms of *Cypripedium* species would not occur without a cold treatment.

Germination Inhibitors. In trying to explain the lack of germination in terrestrial orchid seeds, Withner (1953) suggested the presence of an inhibitor that may be removed by soaking, although no evidence was provided. Kano (1968) presoaked mature seeds of

5 *Cypripedium* species in sterilized nutrient solution for 0, 15 and 45 days before plating on agar plates. Germination and growth of four of the species was poor except for *C. acaule* where germination was best for those cultures soaked for 45 days and poorest in the culture with no soaking. Although the author gave no reasons for this result, Ballard (1987) implied that inhibitory substances were being washed out by prolonged soaking. Fast (1982) recommends soaking as a pretreatment for European terrestrial seeds, based on the assumption that diffusible inhibitors do occur in the testa. The plant growth regulator ABA has been implicated to be this inhibitor (Van der Kinderen 1987). However, Stoutamire (1974) claimed that prolonged soaking in distilled water did not increase germination for several terrestrial orchids (not including *Cypripedium* species). This suggested that diffusible inhibitors in the seed are not an important factor for these species.

Seed Coat Impermeability. Van Waes and Debergh (1986a, 1986b) showed that seed coat impermeability is a major factor affecting germination. Seed coat impermeability has been attributed to the concave shape of the seed which traps air between the seed coat and the embryo (Carlson 1940), and to suberin within the integuments (Harvais 1980). The darker the integuments, the higher the suberization. Germination was increased by treating mature seeds in hypochlorite solutions, which are used for sterilization. Hypochlorite solutions remove suberin in the integuments by oxidation and alkalinisation (Harvais 1980). Removal of the suberin enhances diffusion of water and nutrients into the seed. The optimal duration of the treatment depended upon the species because of

the varying amounts of suberin in the seed coat. For *C. calceolus*, the optimal duration was 4 hours. Longer treatment with hypochlorite solutions resulted in damage to the embryo, whereas shorter treatments did not optimize germination of mature seed (Van Waes and Debergh 1986a, 1986b). These properties have important implications regarding viability tests for terrestrial orchid species which will be discussed later. However, it has also been suggested that pretreatment in hypochlorite solutions results in the leaching out or destruction of germination inhibitors (Linden 1992).

Butcher and Marlow (1989) reported that germination of *C. calceolus* was improved by dissecting the embryo from the seed coat and then soaking in sterile deionized water for 30 to 58 days prior to plating on medium. Miyoshi and Mii (1988) reported that germination of the terrestrial species *Calanthe discolor* was enhanced after ultrasonic treatment, which removed the seed coat. Preliminary success was also achieved by using cell-wall-degrading enzymes to improve germination of *C. calceolus* seed (Linden 1992). However, Stoutamire (1974) contends that seed coat impermeability is not the cause of dormancy in *Cypripedium* species because grinding the seed with sand to abrade the seed coat did not accelerate germination.

Seed Inviability

Stoutamire (1974) proposed that poor germination may simply be the result of seed inviability. This view is also shared by Harvais (1982) and Fast (1982). Seeds may become inviable as they mature or viability may decrease with storage time (Stoutamire 1974; Withner 1953). Seed of rare orchid species is difficult to obtain in European

countries particularly and thus must be stored for long periods of time. When mature seeds are observed under a microscope, it is common to find seeds with shriveled brown embryos within the seed coat (Ballard 1987).

Many of the standard viability tests, such as the tetrazolium chloride test, are ineffective on terrestrial orchid seeds (Harvais 1974; Van Waes and Debergh 1986a, 1986b; Pritchard 1985) although they are effective for tropical, epiphytic orchids. The only real evidence of viability is germination (Harvais 1974). Unfortunately, it can take several months until final germination levels are reached. Even then, unless germination is performed under optimum conditions, it is still difficult to correlate germination with viability. As Harvais (1982) succinctly pointed out, "Since there is no known viability test for orchid seeds, one cannot tell whether in cases of poor germination the seeds are inviable or whether they have a greater requirement for some factor(s)."

Development of a simple fast viability test is extremely desirable, both for research purposes and for commercial production. Recent research has resulted in viability tests for mature seed of European terrestrial species using adaptations of 2,3,5-triphenyl tetrazolium chloride (TTC) (Van Waes and Debergh 1986a, 1986b) and fluorescein diacetate (FDA) (Pritchard 1985) . The differences in response to viability tests between tropical epiphytic orchid species and European terrestrial species has been attributed to differences in seed coat permeability (Van Waes and Debergh 1986a).

The modified TTC test involves 3 steps:

a) Pretreatment of seeds in 5 % calcium hypochlorite (w/v) + 1 % Tween-80 (v/v): the optimal duration of the pretreatment depends on the species.

b) Soak in sterile water for 24 hours.

c) Apply the classical TTC test.

Pretreatment in calcium hypochlorite was necessary to enhance uptake of the stain in the same way that it improves germination. Using the modified TTC test, Van Waes and Debergh (1986b) found that *C. calceolus* seed had 40.2% viability.

The modified FDA test for viability involves:

a) Soaking in distilled water for 16 hours.

b) Rupturing the testa.

c) Applying the FDA test.

Pritchard found that the rapidity of permeation of stain into whole seeds varied with the species. He felt this was partly due to the large airspace between the testa and the embryo in European terrestrial species which hindered access to the stain. For this reason, the seed coat was ruptured. Soaking the seeds prior to rupturing the testa may facilitate the activation of esterase enzymes because it increased the %FDA viability. His study did not include any species of *Cypripedium*.

Culture Conditions

Terrestrial species of orchids, especially *Cypripedium* species, are more difficult to germinate than epiphytic species (Oliva and Arditti 1985; Henrich et al. 1981). This is thought to be because of their greater dependence on the mycorrhizal association in nature compared to that of epiphytic orchids. However, little is known about the exact requirements for the asymbiotic germination of terrestrial orchid seeds including their

requirements for specific macro- and microelements or organic additives during seed germination and protocorm development (Fast 1982; Van Waes and Debergh 1986b).

A large array of media have been used to germinate *Cypripedium* species and these media vary widely in their composition. A compilation of media used for orchid seed germination is given in Arditti (1982) and Fast (1982). Comparisons of some of these media have been carried out (Linden 1980; Oliva and Arditti 1985; Arditti 1984) but Oliva and Arditti (1984) admit that their comparisons provide few answers regarding the germination requirements of North American orchid seeds. Media comparisons are not accurate unless the same seed capsule is used because germination can vary so greatly from capsule to capsule (Harvais 1980; Ballard 1987).

Wide variation in the germination response to any number of media ingredients, including nitrogen source, hormones and complex additives was found. This variation has been attributed to a number of reasons:

1. Terrestrial species have different requirements and therefore different responses than tropical species.
2. There are distinct differences between genera in response to culture conditions (Arditti 1984). Even species within the same genus will respond differently (Henrich et al. 1981).
3. Different stages of maturity of seed may have different nutrient requirements for germination.
4. The apparent response may depend on the stage of germination/development being examined. Therefore, although certain ingredients may be included in a germination medium, they actually have little or no effect on germination but rather optimize subsequent growth of the seedlings.

5. Since media composition can vary widely, germination response to different compounds may depend on the other ingredients in the particular medium tested.

Comprehensive reviews of factors affecting the germination of orchid seeds are given in Arditti (1967) and Arditti and Ernst (1984).

Light vs. Dark Culture Conditions

The majority of studies on terrestrial orchid species concluded that germination is inhibited by light (Harvais 1973; Stoutamire 1974; Allenburg 1976; Linden 1980; Fast 1982; Van Waes and Debergh 1986b; Ballard 1987). Stoutamire (1974) suggested that light-inhibited germination is a protective mechanism which prevents seeds at the soil surface from germinating where they might be subjected to periods of drying. Only those seeds below the soil level may germinate and these are obligately mycorrhizal. Only Oliva and Arditti (1984) contradicted the requirement for darkness during germination. They found that light did not adversely effect germination of *Cypripedium* and other native orchid species.

Mineral Nutrition

Salt-poor Medium. Terrestrial orchid species are generally more sensitive to salt concentration and require a more salt-poor medium than tropical epiphytic orchids (Arditti 1984; Fast 1976 and 1982; Van Waes and Debergh 1986b). In fact some terrestrial species such as *Dactylorhiza* will germinate readily on water and agar

(Stoutamire 1974; Harvais 1972,1974), although they often require a richer medium with a carbohydrate source for further development. However, Harvais (1973) found that *C. reginae* did not germinate on water and agar or on a complete mineral medium. The addition of 1% dextrose to the mineral medium was necessary to achieve germination. This would imply that a carbohydrate source is necessary for germination in this species. Van Waes and Debergh (1986b) showed that germination was best for 14 Western European orchids (not including *Cypripedium*) on a medium containing just microelements, vitamins and sucrose, with no macroelements, but that further protocorm development was arrested. Even slight increases in the level of macroelements decreased germination dramatically, although there were differences among species.

Nitrogen Source. The source of nitrogen appears to be a critical factor in the germination of terrestrial orchid species. Van Waes and Debergh (1986b) have suggested that there is a species requirement for nitrogen. However, many studies have focused on protocorm growth as well as germination. Confusion may have resulted because nitrogen source may increase growth of the protocorm but have little or no effect on germination. Raghavan and Torrey (1964) studied the effect of different N sources on the tropical species *Cattleya*. They found germination and early growth was best on ammonium, regardless of the source, and that seedlings were only able to utilize nitrate ions after 60 days. The ability to use nitrate paralleled the appearance of nitrate reductase in the seedlings. However, they did note that germination would occur on a medium with NO_3- or even in the absence of added N, but that the protocorms would not develop further without ammonium.

Van Waes and Debergh (1986b) studied the effects of different N sources on germination of *Dactylorhiza maculata*. Germination was high on N-free medium but nitrogen was required for later development. Organic nitrogen was preferred and the addition of inorganic N (i.e. KNO_3, $(NH_4)_2SO_4$, or both) decreased germination at all concentrations tested. For germination of *C. calceolus*, Van Waes and Debergh (1986b) suggested a medium containing organic N only. For species of *Orchis*, Mead and Bulard (1979) found that organic N in the form of casein hydrolysate was superior to inorganic N. Addition of ammonium was not necessary but had an improving effect on growth of protocorms.

Complex additives are undefined components commonly added to culture media, such as urea, peptone, casein hydrolysate, potato extract and yeast extract. The exact composition of these components is unknown so exactly what they contribute to the media is uncertain. Some complex additives such as urea, peptone, and casein hydrolysate are considered to be sources of organic nitrogen. Addition of undefined components to culture media may improve germination or growth of tissues but provide little information into specific growth requirements. Comparison of media with complex additives therefore also provides little information.

There are differences in germination response to the source of organic N, regardless of whether it is a defined or undefined component (amino acids, casein hydrolysate, peptone etc.). Results are contradictory and probably depend upon the species, as the following examples may illustrate. Mead and Bulard (1979) found that casein hydrolysate could be replaced by a reconstitution of its amino acids, or glutamine

alone. Van Waes and Debergh (1986b) found that casein hydrolysate and yeast extract did not inhibit germination for *Dactylorhiza maculata* as did other exogenous N sources. L-glutamine and myo-inositol could replace casein hydrolysate. Peptone was inhibitory.

Data are somewhat limited specifically regarding *Cypripedium* species. Harvais (1973) demonstrated that *C. reginae* has a definite requirement for ammonium. Increasing the NH_4NO_3 from 400 mg/L to 1400 mg/L in his potato extract medium was markedly beneficial particularly during the first 10 weeks of germination and growth, resulting in larger protocorms (Harvais 1982). He also noted that the right nitrate/ammonium ratio may be critical. However, he found glutamine to be especially beneficial (compared to glycine and glutamic acid) on a medium containing inorganic N during germination and early growth of the protocorms, resulting in better growth and less protocorm mortality. Harvais (1982) also found that urea was toxic to *C. reginae* seeds and that potato extract was superior to a combination of casein hydrolysate and yeast extract.

Withner (1953) reported that casein hydrolysate was definitely inhibitory for germination of *C. acaule* at 50 mg L^{-1} but peptone was beneficial. Fast (1974) suggested a germination medium for *C. calceolus* that contained peptone and yeast extract.

Hormones. Research results concerning hormones in the culture medium, including gibberellins, cytokinins and auxins, are also inconsistent. Gibberellins generally have a negative or no effect on orchid seed germination, including *Cypripedium* species (Arditti and Ernst 1984). Harvais (1980, 1982) repeatedly found that gibberellic acid in

concentrations of up to 5 mg/L had no effect on the germination and early protocorm development of *C. reginae*. Van Waes and Debergh (1986b) reported that GA_3 did not enhance germination of *C. calceolus*. The only report of a positive effect is cited by Arditti and Ernst (1984). They reported that Borriss (1969) found GA_4 in concentrations of 5 ppm enhanced differentiation of seeds and seedlings of *C. calceolus*.

Cytokinins added to the medium either increase germination of orchid seeds, have no effect, or decrease germination depending on the species. All *Cypripedium* species appear to require cytokinins for germination (Harvais 1982; Van Waes and Debergh 1986b). Although Harvais' (1973) preliminary work found no response to aminopurines, he (1982) later demonstrated that *C. reginae* requires a cytokinin for germination and subsequent growth. He tested three aminopurines, kinetin, benzyl aminopurine (BAP) and 6($\gamma\gamma$-dimethylallylamino)purine (2iP) at three levels. The best hormones for germination and early growth were 2iP at concentrations of less than 0.25 mg L^{-1} followed by BAP at 0.25-0.5 mg L^{-1} and kinetin at less than 0.5 mg L^{-1}. However, later development (greening, growth and survival) of plantlets was favored by kinetin, BAP and 2iP in that order. For this reason Harvais adopted kinetin in subsequent studies. Van Waes and Debergh (1986b) also demonstrated that *C. calceolus* requires a cytokinin. At a low concentration (0.88 μM), BA improved germination of mature seed.

Auxins, in general, do not enhance orchid seed germination (Arditti and Ernst 1984). Harvais (1982) demonstrated that auxins (IAA, NAA, IBA, and 2,4-D) did not stimulate germination of *C. reginae*, but, in combination with kinetin, NAA in particular improved growth and survival of protocorms. For this reason, his potato extract medium included kinetin at 1 mg L^{-1} and NAA at 0.1 mg L^{-1} (i.e, 10:1 ratio).

CHAPTER 1

In vitro germination of three *Cypripedium* species in relation to time of seed collection, media and cold treatment

Abstract

In order to maximize the *in vitro* seed germination of three *Cypripedium* species, *C. candidum* Muhl. ex Willd., *C. reginae* Walt., and *C. calceolus* L. var. *parviflorum* (Salisb.) Fern., the optimum time of seed collection and the suitability of various media were studied. The effects of a cold treatment were also investigated for *C. candidum*. Seed pods were collected at regular intervals after pollination, these ranged from green pods at 5 weeks to dry undehisced pods at 12 weeks. Seed was inoculated onto three media, Harvais, Van Waes and Debergh, and a modified Norstog and germination was assessed at 4-week intervals for 20 weeks. *Cypripedium reginae* germinated better than the other two species. Except for *C. candidum* in 1989, when germination peaked for seed collected at 6 weeks after pollination, seed collected at 8 weeks after pollination had the highest germination. After 8 weeks, germination decreased sharply in all species, dropping below 1% for *C. candidum*. There were differences in germination among media but the effects on germination were not consistent and depended on the year, the date of collection, and the species. Subsequent development of protocorms was superior in all cases on the modified Norstog medium. The effect of cold treatment on germination of *C. candidum* seeds was variable and therefore its importance is questionable.

Introduction

The *Cypripedium* species are north temperate terrestrial orchids commonly called lady's-slippers because of the slipper-like appearance of the flower. Lady's-slippers are becoming increasingly rare due to destruction of their native habitat by human encroachment (Bowles 1983; Brownell 1981). *Cypripedium candidum* Muhl. ex Willd. is considered to be endangered in Canada, including the Province of Manitoba. Because of their delicate flower, *Cypripedium* species show potential in the nursery trade and plants currently sold by nursery growers are usually taken from wild stands. Therefore, practical, efficient methods of propagation are necessary to prevent depletion and possible extinction of natural stands.

The propagation method that has received the most attention for *Cypripedium* species is *in vitro* seed germination. However, the success of such methods has been variable. In nature, an association with endophytic fungi is necessary for germination and growth and it is this dependence that may make *in vitro* culture difficult (Hadley and Pegg 1989). Germination is often poor, slow and erratic. Results of germination studies have been contradictory and in many cases, somewhat empirical in nature, with limited control of variability. For example, seeds at different stages of maturity have been used within the same study (Linden 1980; Henrich et al. 1981; Oliva and Arditti 1984). Many media, varying widely in composition, have been used (Arditti et al. 1982) but it is difficult to determine which is the most appropriate because of species and genotypic variability (Henrich et al. 1981). Little is known about *in vitro* requirements for

germination, including specific requirements for macro- and microelements (Fast 1982). It is also important to distinguish between requirements for germination and requirements for protocorm growth following germination. Certain substances may improve growth of protocorms but have little or no effect on germination, or vice versa.

Since Withner's (1953) report of improved germination with immature seed compared with mature seed in *C. acaule*, subsequent studies have revealed similar findings for other *Cypripedium* species (Stoutamire 1964; Light 1989; St. Arnaud et al. 1992). In contrast, in *C. reginae*, Ballard (1987) reported unpredictable variation in the germination response as the seed matured. Despite these general observations, the optimal time to collect immature seed to maximize germination is not known.

The superior germination typical of immature seed suggests that a type of dormancy may exist in the mature seed. It has been reported that a cold treatment may be required to break dormancy (Stoutamire 1974; Fast 1982; Ballard 1987) but again the results have been contradictory (Van Waes and Debergh 1986b; Light 1989). It should be pointed out that cold treatment to break seed dormancy is different from a cold treatment to break 'epicotyl dormancy' (Curtis 1943) in plantlets.

Because of the lack of carefully controlled research, an investigation of germination of three native *Cypripedium* species was initiated with the following objectives: (i) to determine the optimum time to collect seed to maximize germination and quantify decreased germination capabilities as the seed matures, (ii) to compare the effects of three media on germination, and (iii) to investigate the effect of a cold treatment on germination for *C. candidum* seeds.

Materials and Methods

Collection sites

Experiments were established using seed from Manitoba populations of three *Cypripedium* species: *C. calceolus* L. var. *parviflorum* (Salisb.) Fern. (Beausejour); *C. reginae* Walt. (Winnipeg); and *C. candidum* Muhl. (Winnipeg and Kleefeld). Two populations were used for *C. candidum* in one of the experiments because pod formation was low for this species in that year.

Pollination

A large number of flowers from each species were self-pollinated manually, using the wide end of a toothpick to transfer both of the sticky pollinia onto the column of the same flower (Harvais 1980). Only those flowers with 2 intact pollinia were used as this indicated that they had not been previously visited by an insect pollinator (Plowright et al. 1980). Successful pollination by insects is quite low and pod formation is pollinator limited (Davis 1986; Bowles 1983; Brownell 1981). Hand-pollination ensures good seed set, results in a greater number of seeds per pod, and provides increased seed viability and germination (Harvais 1980; Ballard 1987).

Experimental design

Two *in vitro* experiments were established. In each experiment, pods were randomly collected at regular intervals after pollination (Fig. 1.1). Seeds from each pod

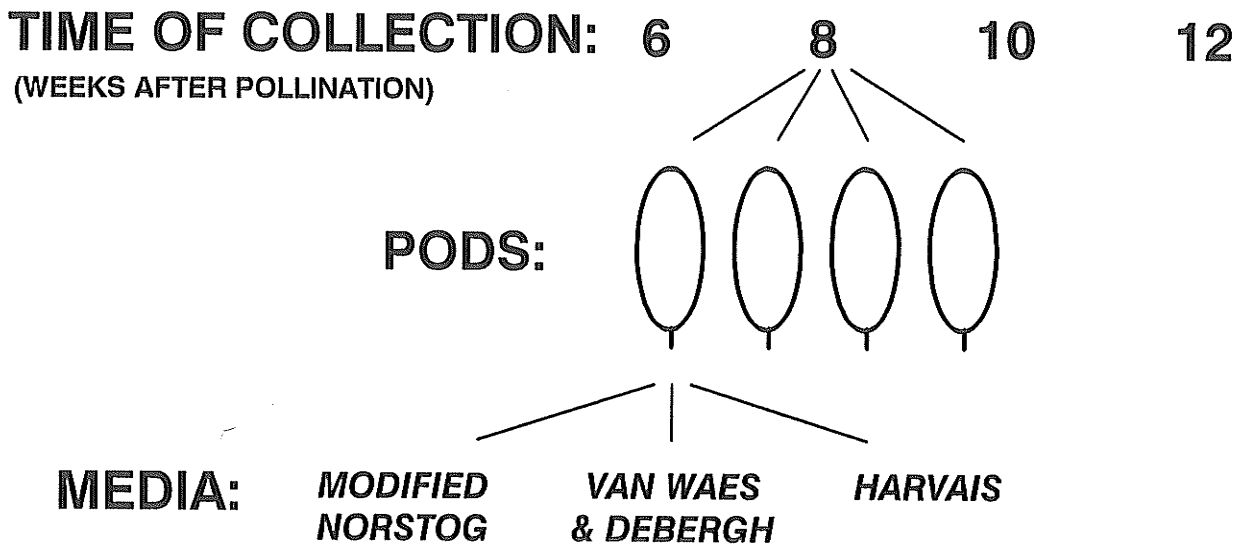


FIG. 1.1. The general design for experiment 1, showing the relationship between media, pods and time of seed collection for the *in vitro* germination studies of *Cypripedium* species. In experiment 2, the time of collection was expanded to include 5 and 7 WAP and a cold treatment was added (see text for details).

were divided into lots for inoculation onto three media. In experiment 1, there was one plate for each medium and plates were placed at room temperature only. In the second experiment, seeds were placed in either a cold treatment or at 23°C for all media. There were 2 plates for each treatment combination.

Seed collection

In experiment 1, four pods of each species were collected at 6, 8, 10 and 12 weeks after pollination (WAP) in 1989 (Fig. 1.1). In experiment 2, four pods of *C. candidum* only were collected at 5, 6, 7, 8, 10 and 12 WAP in 1990. The pods were taken to the laboratory for inoculation of the seeds onto media.

Media

In both experiments, three media were compared: Harvais (1982), Van Waes and Debergh (VWD) (1986b) and a modified Norstog (Fig. 1.1). Norstog medium (1973) was modified as follows: the macroelements were reduced by half, the pH of the medium was adjusted using 5M NaOH, instead of NH_4OH, and benzyl adenine was added at 0.2 mg L^{-1}. Modified Norstog is a completely defined medium whereas the other 2 media contain complex additives (Appendix A).

Culture conditions

In experiment 1, all of the petri dishes were placed in an incubator at 23°C in the dark. In experiment 2, one-half of the petri dishes were placed at 23°C in the dark, and the other half were placed at 4°C for 2 months, after which time they were also placed at 23°C in the dark.

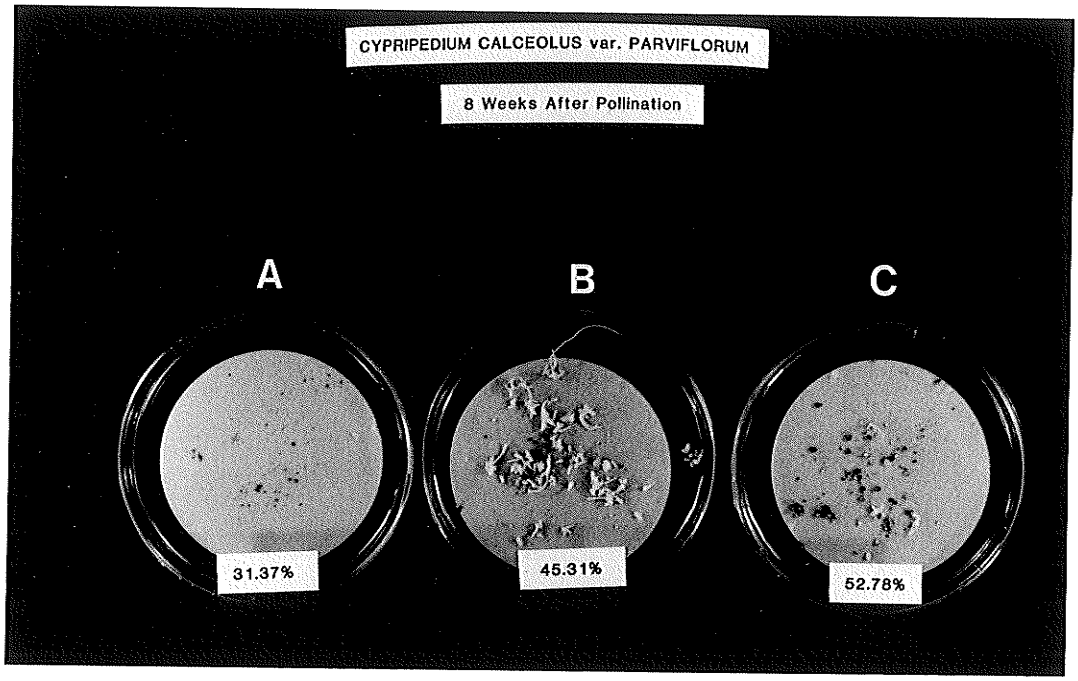
Inoculation

Working in a laminar flowhood, pods were surface sterilized in a 2% sodium hypochlorite solution for 15-20 minutes and then rinsed three times in distilled deionized water. A piece of sterile filter paper was placed on top of the medium in each petri dish prior to inoculation. Each pod was cut open and the seeds were either scooped out with forceps or shaken directly onto the filter paper (Fig. 1.2). Each plate contained approximately 150 to 300 seeds. The filter paper facilitated the transfer of seeds onto fresh media at 6 week intervals. This was considered to be important to prevent desiccation of the media and accumulation of phenolic compounds.

Germination

Each plate was examined at 4-week intervals for 20 weeks in culture using a Zeiss stereomicroscope (10X magnification) and the number of germinating seeds recorded. Germination was considered to have occurred when the embryo emerged from the testa. Germination was expressed as a percentage of the total number of seeds inoculated. The germinated embryo is called a protocorm (Arditti 1967). Initially, the protocorm is just a small round mass with little tissue differentiation. As the protocorm develops, it assumes a cone shape, so that a basal and an apical meristematic region are established.

FIG. 1.2. Protocorm development of *C. calceolus* var. *parviflorum* after 20 weeks in culture on 3 media showing percent germination. A. Harvais medium, B. modified Norstog medium, and C. Van Waes and Debergh medium. Seed was collected at eight weeks after pollination. Dimensions of petri dishes, 100 x 15 mm.


CYPRIPEDIUM CALCEOLUS var. PARVIFLORUM
8 Weeks After Pollination
A
B
C
31.37%
45.31%
52.78%

Statistical Analysis

Analysis of variance (ANOVA) was performed separately for each species on the final germination counts after 20 weeks in culture. The model included time of collection (WAP), pods nested within time of collection and media. In Experiment 2, cold treatment was added to the model. Germination percentages were transformed using the arc sin square root to overcome heteroscedasticity (Steel and Torrie 1980).

Results

Pod and Seed Development

Pods collected at 5 and 6 WAP were green and the seeds were white and moist. At this stage, part of the placenta remained attached to the seeds during inoculation. By 8 WAP, the seeds were beginning to turn light brown, so there was a mixture of white and brown seeds in the same pod. At this time, the seeds were dry and loose, and could be readily shaken onto the petri dish. Complete maturity of seeds and pods appeared to be achieved by 12 WAP and for *C. reginae* and *C. calceolus* var. *parviflorum*, the pods were close to dehiscing. In *C. candidum*, two of the four pods collected in 1989 at 12 WAP were still green although the seeds inside were dark brown.

In vitro Germination

Comparison of Species

Regardless of treatment, germination of *C. reginae* seed was highest while that of the other two species was relatively similar (Figs. 1.3 to 1.5). However, the variability in germination was also greater in *C. reginae*. Following germination, there were also differences in protocorm development among species. Protocorms of *C. reginae* developed more rapidly, producing many long roots sooner than the other two species.

Time of Seed Collection

Experiment 1. In 1989, the time after pollination that seed was collected had a significant effect on germination in all 3 species (Figs. 1.3 to 1.5). In *C. reginae* (Fig. 1.3) and *C. calceolus* var. *parviflorum* (Fig. 1.4), germination peaked for seed collected at 8 WAP. However, with modified Norstog and VWD, there was little difference in germination between seed collected at 6 or 8 WAP. After 8 WAP there was a sharp decrease in germination. In *C. candidum* (Fig. 1.5), the germination peak occurred for seed collected at 6 WAP, the initial inoculum date, after which time germination decreased sharply. There was an increase in germination with seed collected at 12 WAP compared to 10 WAP, but only on VWD medium. The reasons for this increase are unknown.

Experiment 2. In 1990, the time of seed collection again had a significant effect on germination in *C. candidum* (Fig. 1.6) but the pattern was somewhat different than in the previous year. Beginning at 5 WAP, germination gradually increased until 8

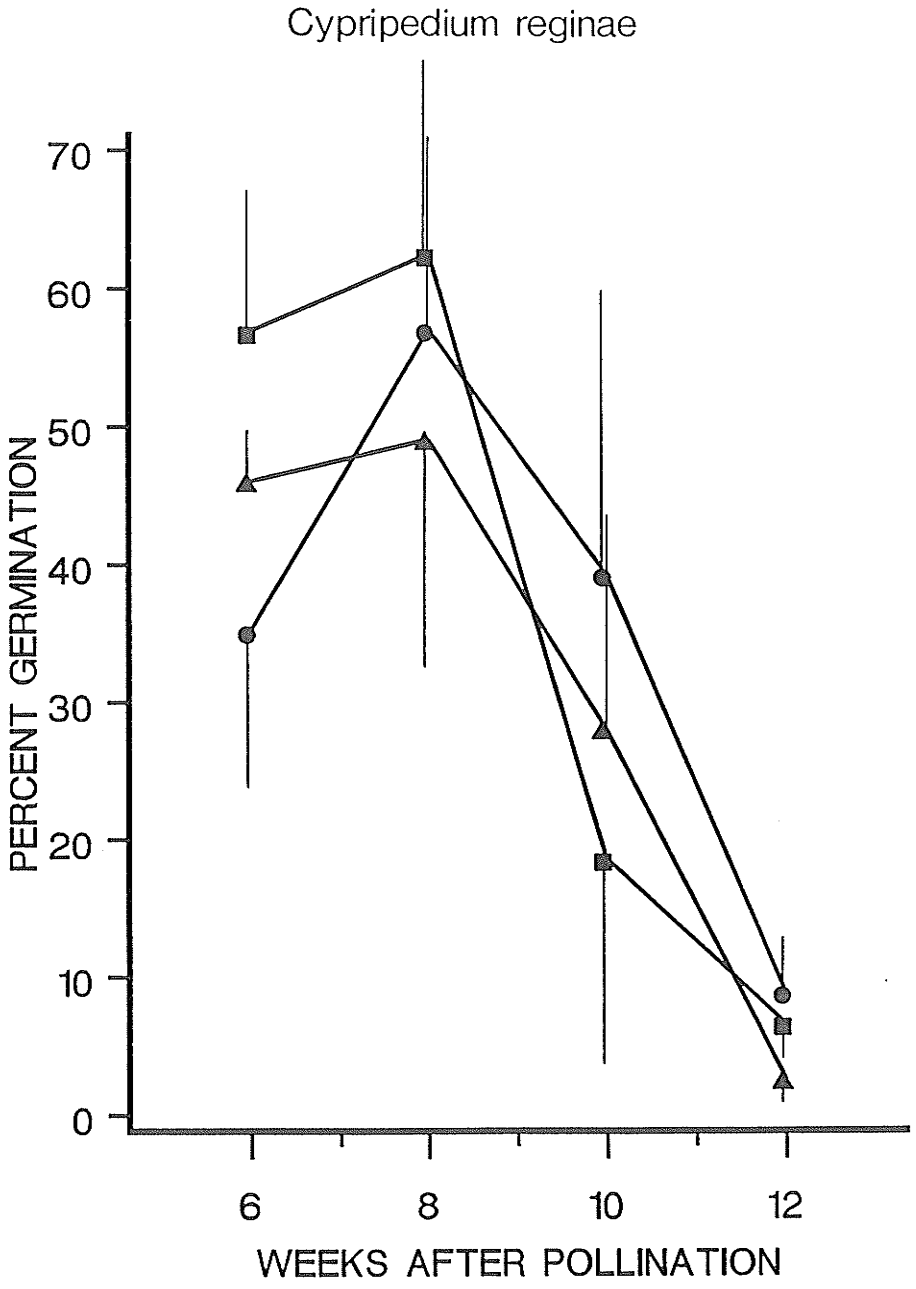


FIG. 1.3. Mean percent germination of *C. reginae* seed collected at successive weeks after pollination on modified Norstog medium (■), Van Waes and Debergh medium (▲), and Harvais medium (●) after twenty weeks in culture. From ANOVA, significance levels were: time of collection, p=0.0370, media, p=0.1679, time of collection x media interaction, p=0.4986. Vertical lines indicate standard error. Those points without error bars had standard errors smaller than the dimensions of the symbol.

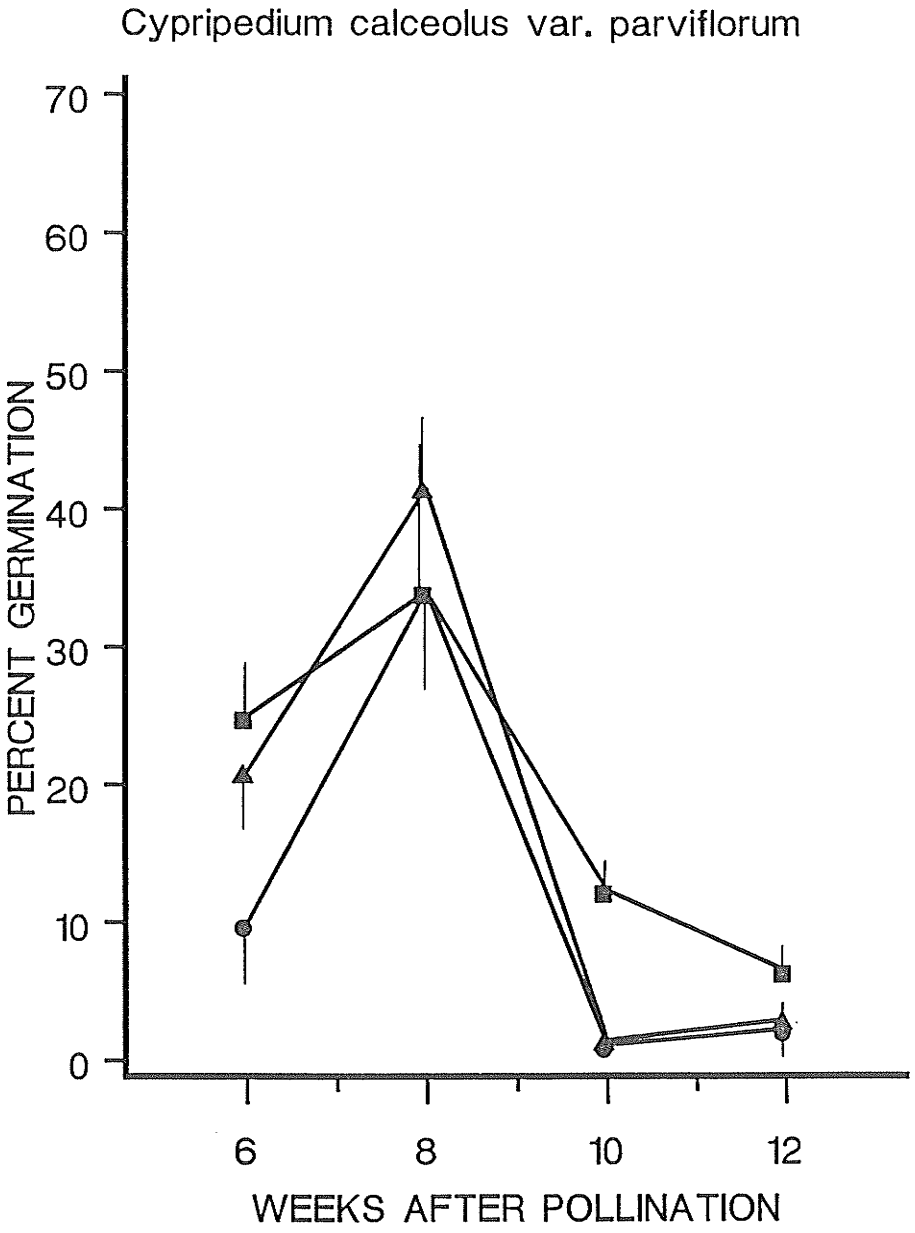


FIG. 1.4. Mean percent germination of *C. calceolus* var. *parviflorum* seed collected at successive weeks after pollination on modified Norstog medium (■), Van Waes and Debergh medium (▲), and Harvais medium (●) after twenty weeks in culture. From ANOVA, significance levels were: time of collection, $p<0.0001$, media, $p<0.0001$, time of collection x media interaction, $p=0.0454$. Vertical lines indicate standard error. Those points without error bars had standard errors smaller than the dimensions of the symbol.

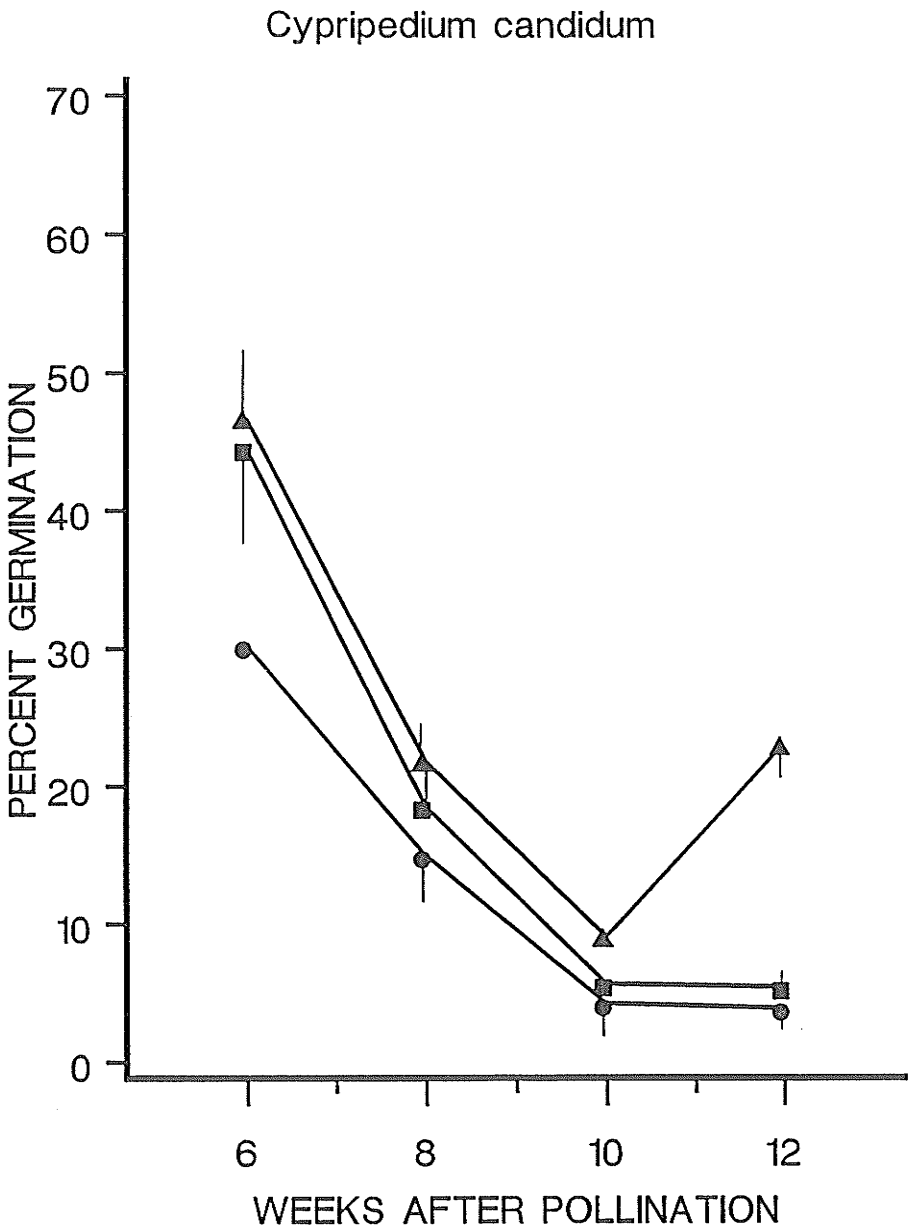


FIG. 1.5. Mean percent germination of *C. candidum* seed collected in 1989 at successive weeks after pollination on modified Norstog medium (■), Van Waes and Debergh medium (▲), and Harvais medium (●) after twenty weeks in culture. From ANOVA, significance levels were: time of collection, $p<0.0001$, media, $p<0.0001$, time of collection x media interaction, $p=0.0124$. Vertical lines indicate standard error. Except for Norstog and VWD media at 10 WAP in which there was only one value due to contamination, those points without error bars had standard errors smaller than the dimensions of the symbol.

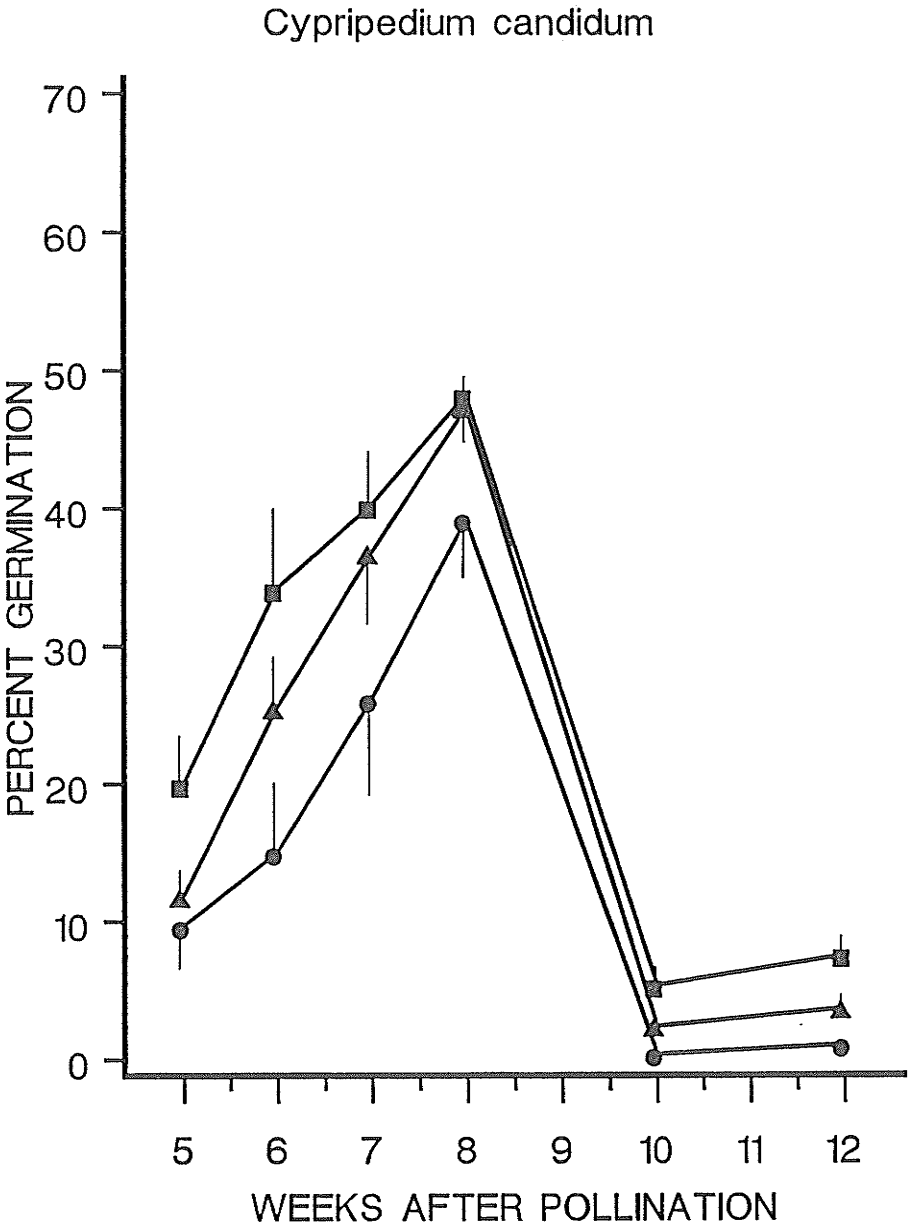


FIG. 1.6. Mean percent germination of *C. candidum* room temperature treated seed collected in 1990 at successive weeks after pollination on modified Norstog medium (■), Van Waes and Debergh medium (▲), and Harvais medium (●) after twenty weeks in culture. From ANOVA, significance levels were: time of collection, $p=0.0009$, media, $p<0.0001$, time of collection x media interaction, $p=0.7131$, cold treatment $p=0.0263$, time of collection x treatment $p=0.0176$, media x treatment $p=0.0056$. Vertical lines indicate standard errors. Those points without error bars had standard errors smaller than the dimensions of the symbol, except for Norstog medium at 5 WAP in which there was only one value due to contamination.

WAP, as it did for the other two species in the previous year, followed by a sharp decrease at 10 WAP. There was little difference in germination between seed collected at 10 and 12 WAP.

Media

Experiment 1. In *C. reginae*, there were no significant differences in germination between media (Appendix B, Table B-1). In *C. calceolus* var. *parviflorum* and *C. candidum* there was a significant interaction between media and time of seed collection. Separate ANOVAs performed for each time of collection revealed that in *C. calceolus* var. *parviflorum* there were no significant differences between media at 8 WAP but at 10 and 12 WAP germination was highest on modified Norstog compared to the other two media (Appendix B, Table B-2) . In *C. candidum*, germination on Harvais was lowest at 6 WAP and germination on VWD was greatest at 12 WAP (Appendix B, Table B-3).

Experiment 2. In 1990, there were significant differences in the germination of *C. candidum* seeds among media. The pattern in relation to WAP was similar on all three media (Fig. 1.6). In contrast to 1989, there was no interaction between WAP and media. Based on separate ANOVAs for each time of collection, germination on modified Norstog was significantly higher than on Harvais ($p<0.01$), except for 5 and 6 WAP ($p=0.07$ and $p=0.03$, respectively) (Appendix B, Table B-4).

Following germination, protocorm development varied with the media (Fig. 1.2). Protocorm development was faster on modified Norstog for all 3 species, and at all times of collection. By 20 weeks in culture, many protocorms had one or more long roots. Conversely, on VWD medium, protocorms were small with a few roots. With the

exception of *C. reginae*, protocorms on Harvais medium were small with no root formation, and many had turned black.

Cold Treatment

Experiment 2. The effects of cold treatment on germination were variable and depended on both the media and the time of collection (Fig. 1.7). In general, the cold treatment advanced the overall pattern, so that germination peaked at 7 WAP, as opposed to 8 WAP (compare Figs. 1.6 and 1.7). At the peak, germination was greatest on modified Norstog medium with a cold treatment compared to Norstog at 23°C. With Harvais medium, germination was considerably lower after the cold treatment than at 23°C. At 12 WAP, as the seeds approached maturity, germination of cold treated seeds was greater than those held at 23°C but only on modified Norstog and VWD media.

Rate of Germination

Experiment 2. The progression of germination over 20 weeks in culture was compared for *C. candidum* seed collected at various times after pollination on modified Norstog medium (Fig. 1.8), VWD medium (Fig. 1.9) and Harvais medium (Fig. 1.10). There were differences in the pattern of germination over time between seed collected at different times after pollination for all media. These differences were evident after just 4 weeks and were maintained throughout the culture period. Germination was higher with seed collected at 8 WAP than at the other times of collection during the entire time in culture on all 3 media.

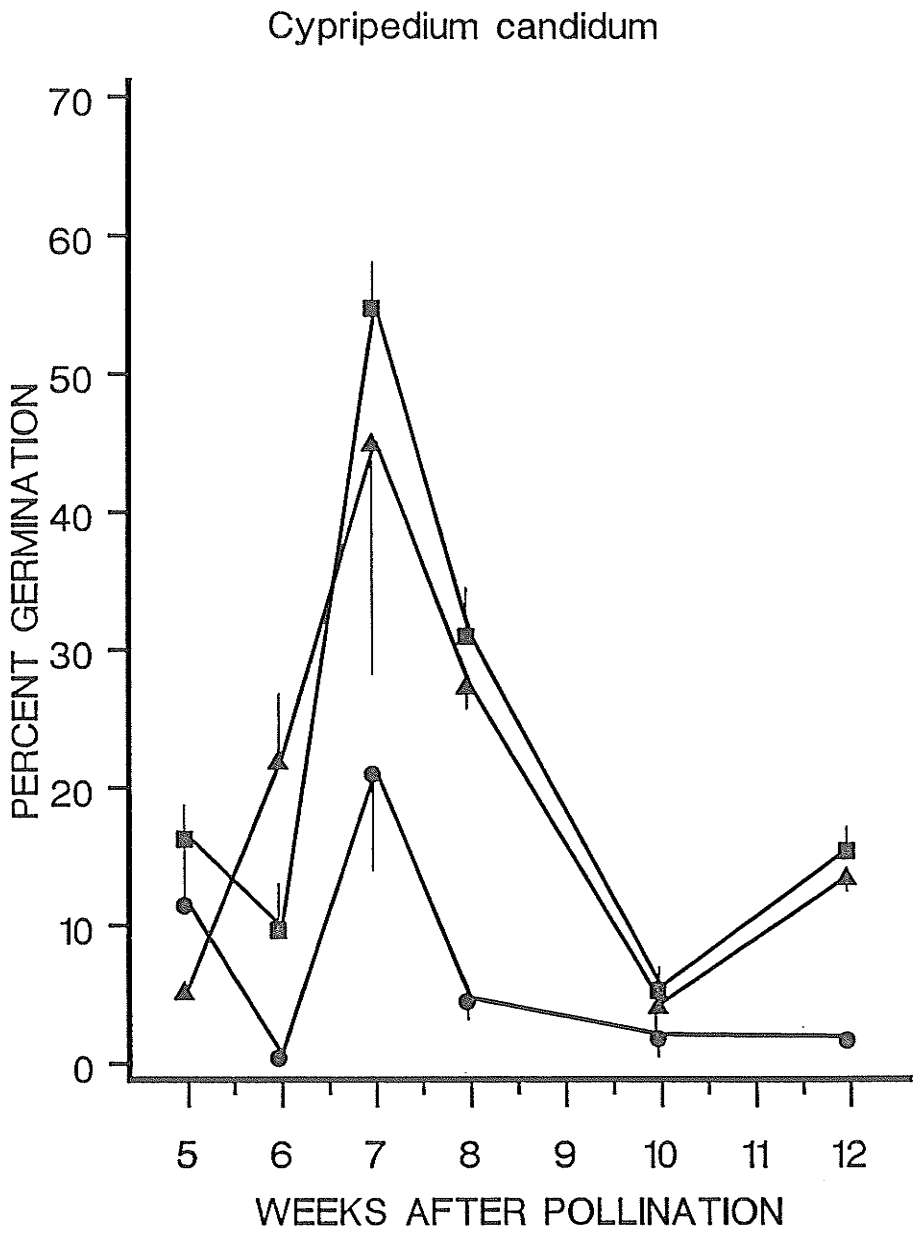


FIG. 1.7. Mean percent germination of *C. candidum* cold treated seed collected in 1990 at successive weeks after pollination on modified Norstog medium (■), Van Waes and Debergh medium (▲), and Harvais medium (●) after twenty weeks in culture. From ANOVA, significance levels were: time of collection, $p=0.0009$, media, $p<0.0001$, time of collection x media interaction, $p=0.7131$, cold treatment $p=0.0263$, time of collection x treatment $p=0.0176$, media x treatment $p=0.0056$. Vertical lines indicate standard errors. Those points without error bars had standard errors smaller than the dimensions of the symbol.

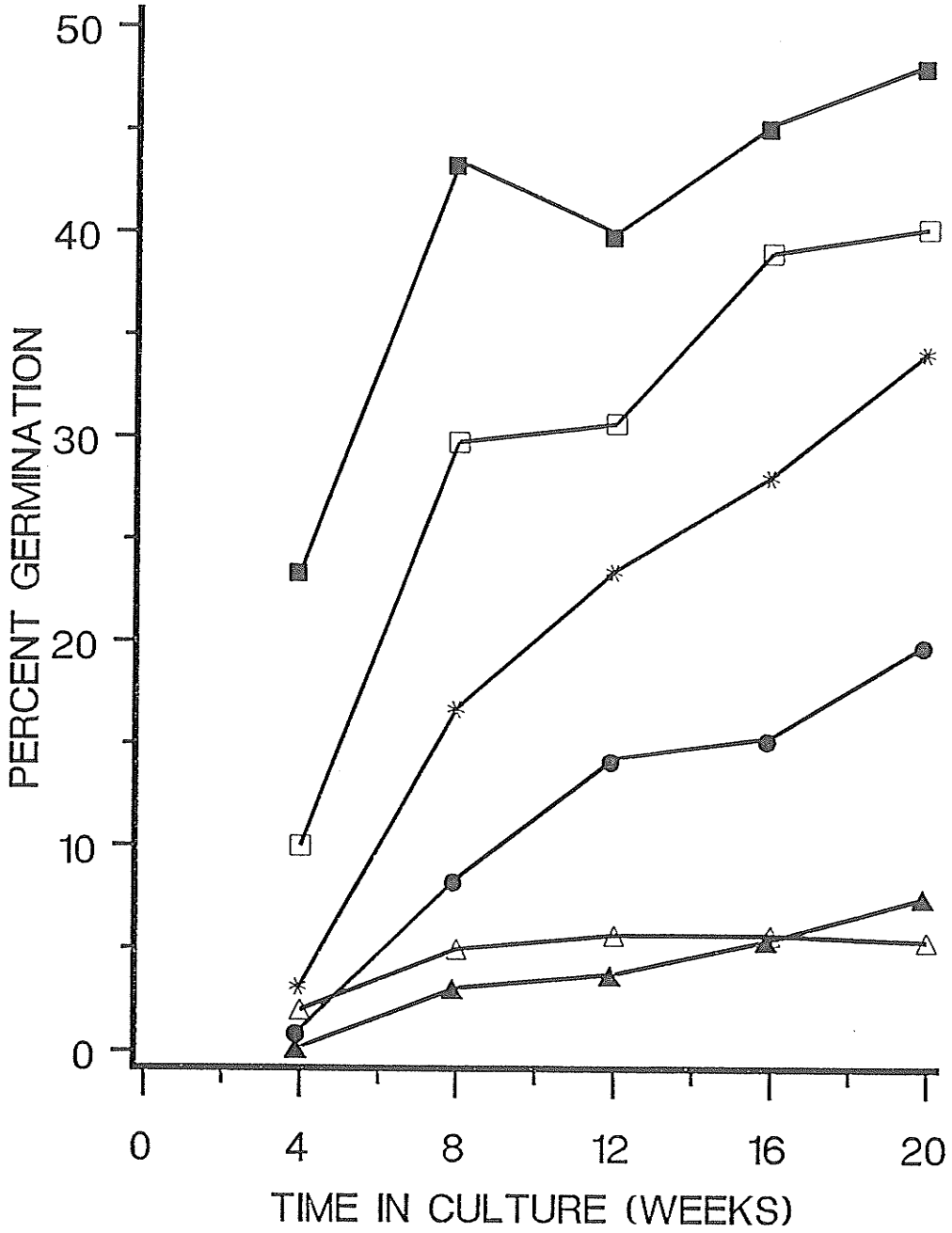


FIG. 1.8. Mean percent germination of *C. candidum* on modified Norstog medium in relation to time in culture for seed collected in 1990 at 5 WAP (●), 6 WAP (*), 7 WAP (□), 8 WAP (■), 10 WAP (Δ) and 12 WAP (▲).

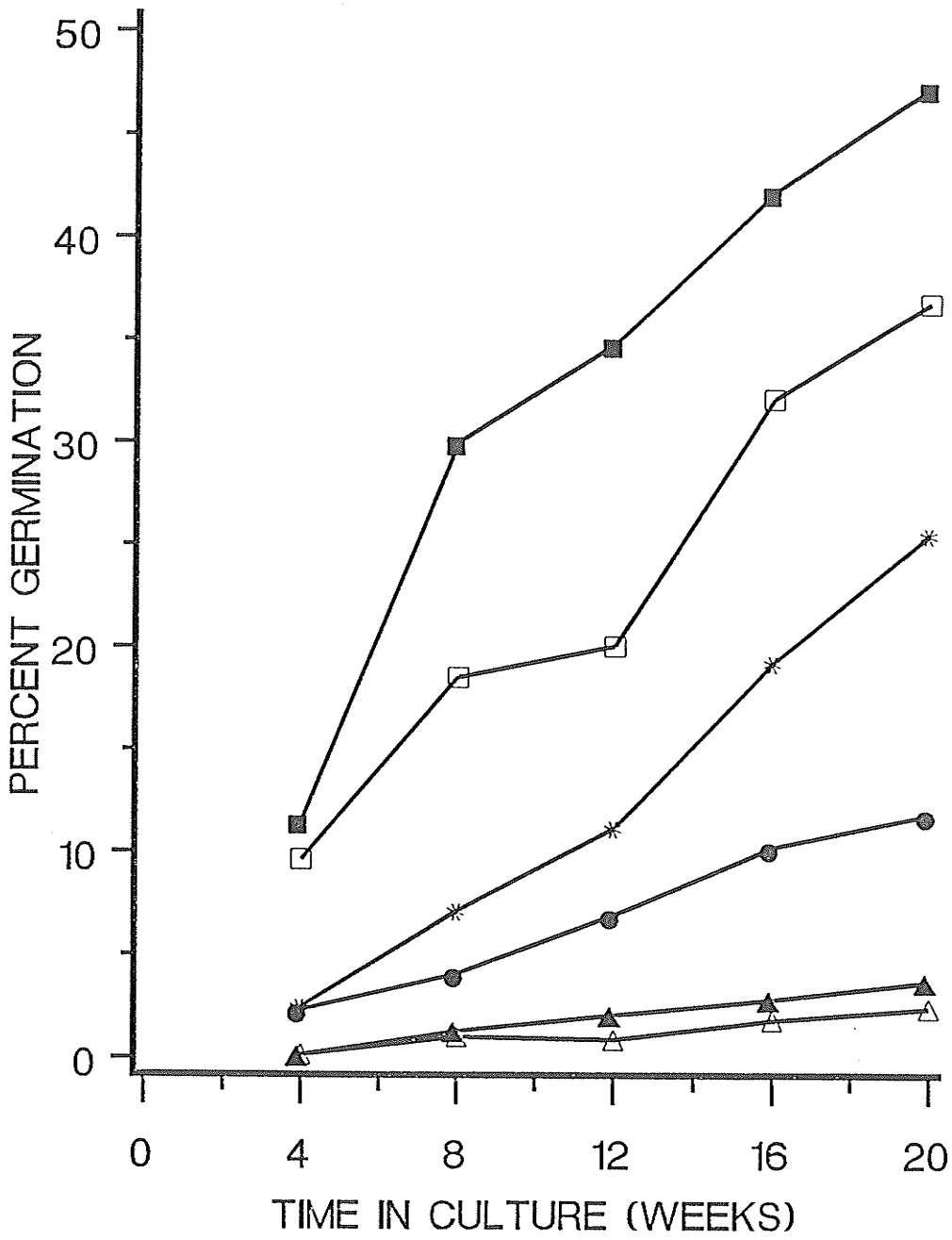


FIG. 1.9. Mean percent germination of *C. candidum* on Van Waes Debergh medium in relation to time in culture for seed collected in 1990 at 5 WAP (●), 6 WAP (*), 7 WAP (□), 8 WAP (■), 10 WAP (Δ) and 12 WAP (▲).

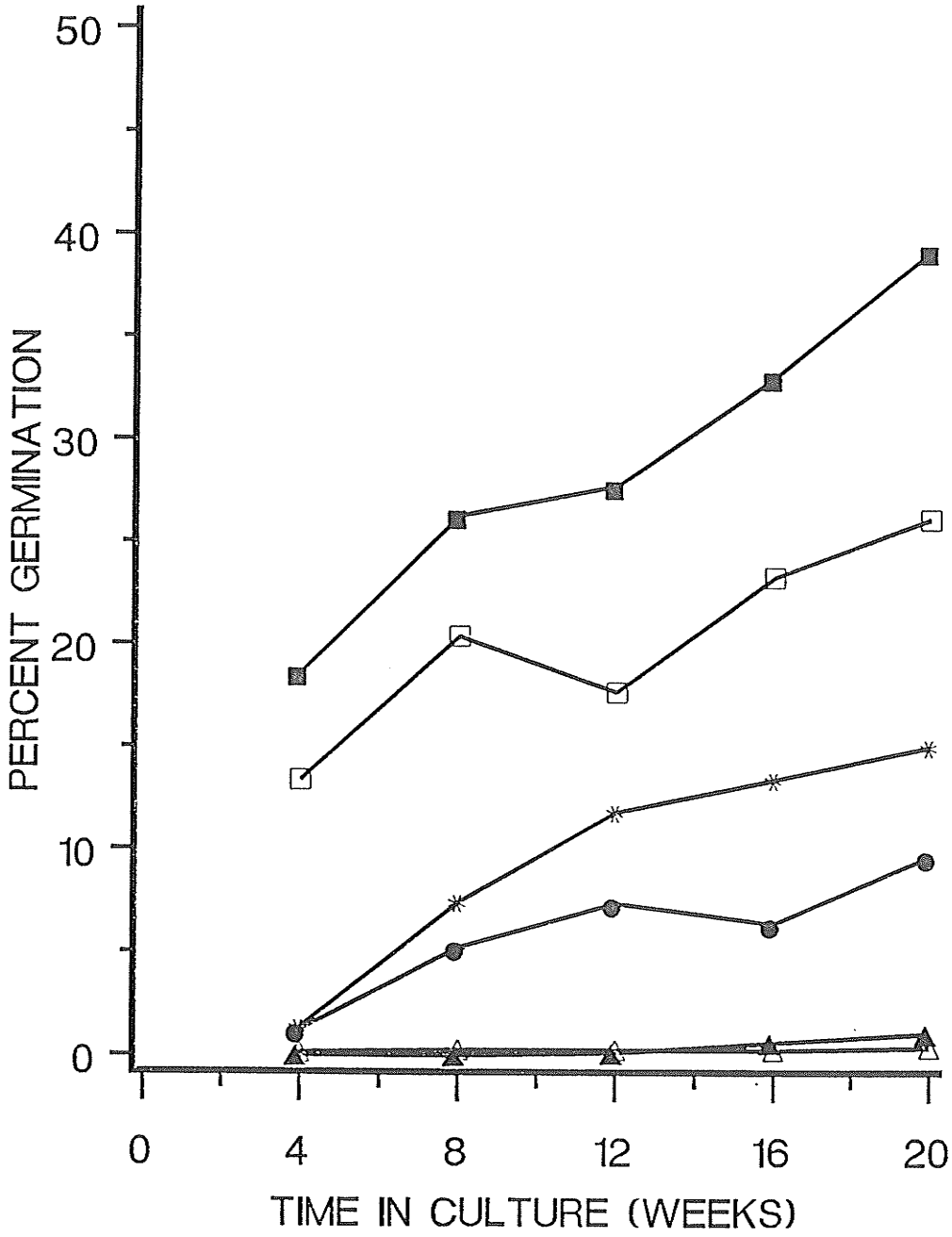


FIG. 1.10. Mean percent germination of *C. candidum* on Harvais medium over time in culture for seed collected in 1990 at 5 WAP (●), 6 WAP (*), 7 WAP (□), 8 WAP (■), 10 WAP (Δ) and 12 WAP (▲).

Despite general similarities in the pattern of germination in relation to the time in culture, there was variation in the rate of germination between the different times of seed collection and the three media. In general, seed collected at 7 and 8 WAP showed a dramatic increase in germination between 4 and 8 weeks in culture (Figs. 1.8 to 1.10). On modified Norstog, germination after 8 weeks in culture was approaching the maximum reached at 20 weeks. In contrast, seed collected at 5 and 6 WAP showed a relatively constant increase in germination rate during the entire time in culture for all media. Seed collected at 10 and 12 WAP showed only small increases in germination during the 20 weeks in culture, although the increase was greater on modified Norstog than the other two media.

In several cases, the percent germination decreased between 8 and 12 weeks in culture. Many protocorms which had just emerged from the testa at 8 weeks turned black and died, so that by 12 weeks they were no longer counted as germinated (St. Arnaud et al. 1992).

Discussion

Numerous studies have shown that immature seed of *Cypripedium* species germinates better than mature seed (Withner 1953; Stoutamire 1964; Fast 1974; Harvais 1980; Light 1989; St. Arnaud et al. 1992) but few rigorously controlled experiments have been conducted to determine if there is an optimum time to collect seed to maximize germination. In the present investigation, an optimum time of about 8 weeks after

pollination was found for all 3 species. In contrast, Ballard (1987) found no such optimum in *C. reginae* for seed collected 35 to 95 days after pollination. Although a wide range of developmental stages were tested, there was little replication. Light (1989) suggested that natural variation in pod development may affect the determination of an optimum time for collection. Based on a study correlating pod dimensions with internal development (Duncan and Curtis 1942), Light (1989) used pod diameter in *C. calceolus* var. *pubescens* to determine that harvesting at 40 to 55 days after pollination optimized germination. Variation in pod development may account for the differences in the optimum time for germination in *C. candidum* between sites and years found in the present study.

The determination of an optimum stage of development for *in vitro* seed germination suggests that, prior to this stage, the seed may be too immature and has not reached a state of competence for germination. Light (1989) showed that embryos of *C. calceolus* var. *pubescens* collected at 43 days post-pollination consisted of only 3 cells. Ballard (1987) found that seed of *C. reginae* collected prior to 45 days after pollination usually gave the lowest germination percentage. Such a delay in seed maturation may be related to the slow fertilization process in orchids. Duncan and Curtis (1942) found that actual fertilization was not complete in *C. pubescens* until 5 weeks after pollination. In *C. parviflorum*, Carlson (1940) showed that fertilization occurred between 26 and 33 days after pollination. Therefore seed collected at 5 and 6 WAP, although capable of some limited germination, is still in a rudimentary form and fertilization of most ovules may have occurred just prior to pod collection. The embryos may not be capable of

continued growth once placed on the medium or they may require more stringent culture/media conditions.

For seed collected after 8 WAP, there was a sharp decrease in germination for all 3 species under the *in vitro* conditions used. It has been reported that increases in the hydrophobic nature of the seed coat during development may inhibit germination of mature seed of terrestrial orchid species (Harvais 1980; Van Waes and Debergh 1986a, 1986b). In the present study, as the seed matured, the seed darkened in color. This change in color is a function of the amount of suberin in the seed coat (Harvais 1980). More suberization leads to darker integuments and contributes to the hydrophobic nature of the mature seed. Hypochlorite solutions, when used to sterilize seeds individually, remove the suberin in the integuments so that germination of mature seed is enhanced (Harvais 1980; Van Waes and Debergh 1986a, 1986b).

Other suggested causes for poor germination of mature seed include lack of viability (Van Waes and Debergh 1986b), presence of germination inhibitors in the mature seed coat (Fast 1974), and onset of dormancy in the mature seed (Stoutamire 1974). For *C. calceolus*, viability of mature seed as low as 40.2% has been reported (Van Waes and Debergh 1986b). Low viability may partially explain the relatively low germination observed at the optimum time of seed collection for all species examined in the present study.

Cold treatment or stratification is often required to break dormancy in seeds of many temperate perennial species. Cold treatment produced contradictory results with *Cypripedium* species. Several reports have suggested a need for a cold treatment (Fast 1982; Ballard 1987). Ballard (1987) reported that a cold treatment of at least 2 months

is absolutely necessary for germination of *C. reginae* and suggests that dormancy is established almost as soon as the capacity for germination is developed. Adequate germination and growth without the use of cold treatment has also been reported (Harvais 1973; Henrich et al. 1981) and Van Waes and Debergh (1986b) and Light (1989) found that a cold treatment actually decreased germination of *C. calceolus* and *C. calceolus* var. *pubescens*. The use of a cold treatment with *C. candidum* in this research provided no clarification to these contradictions. Except for the slightly higher germination at the optimum time of seed collection, the cold treatment did not increase germination enough to warrant the 2-month delay involved. In fact germination was decreased on Harvais medium at certain times of seed collection. If only mature seed is available, the increase in germination after cold treatment on two of the media may justify its use but this would require further investigation.

Cypripedium reginae appears to be more amenable to culture than the other two species. Higher germination numbers were attained with *C. reginae* and the protocorms were more vigorous. This is consistent with other reports (Oliva and Arditti 1984) and may be the reason why *C. reginae* has been the focus of more studies than other *Cypripedium* species (Harvais 1973; Ballard 1987; Frosch 1986).

Because the effect of media on germination was not significant in *C. reginae*, the composition of the medium may not be as critical a factor affecting germination in *C. reginae* as in the other two species. Variation in the germination response was greatest in *C. reginae*. This may be related to high pod-to-pod variability, which has been reported in *Cypripedium* species, especially with *C. reginae* (Harvais 1982; Ballard 1987).

Harvais medium (1982) proved to be as effective for the germination of *C. reginae* seed as the other two media tested, but that was not the case for the other species examined. This is not surprising since Harvais perfected his medium specifically for *C. reginae*. He suggested that it could be an appropriate medium for germination of other terrestrial orchid species. However, Fast (1976) found that *C. calceolus* germinated better on 1/4-strength Harvais medium.

Norstog (1973) described a medium specifically formulated for culturing immature excised barley embryos. Henrich et al. (1981) proposed that the Norstog medium might be appropriate for germination of terrestrial orchid species because these seeds are essentially rudimentary undifferentiated embryos containing no endosperm. However, their investigation of several *Cypripedium* species revealed relatively low germination and considerable variability among the species using this medium and suggested the need for refinement of the Norstog medium. It has been shown that a salt-poor medium, an inorganic nitrogen source and the addition of a cytokinin are critical for the germination of *Cypripedium* species (Harvais 1982; Van Waes and Debergh 1986b). The modifications to Norstog medium in the present study were favorable for both the germination of 3 *Cypripedium* species and for the continued growth of the protocorms. In addition, the rate of germination supported was greater, especially for seed collected at 8 WAP, than was the case in the other media. Norstog medium is also suitable for future studies into the specific requirements of germination and protocorm growth of *Cypripedium* species and other terrestrial orchids because it is a completely defined medium.

CHAPTER 2

The cytokinin preference

for *in vitro* germination and protocorm growth

of *Cypripedium candidum*

Abstract

Cytokinin preference and optimum cytokinin levels were investigated for *in vitro* germination and protocorm growth of *Cypripedium candidum* Muhl. ex Willd. using seed collected eight weeks after pollination. Benzyl adenine (BA) and 6-(α,α-dimethylallylamino)-purine (2iP) in concentrations of up to 0.8 mg L^{-1} enhanced germination significantly compared to the control without cytokinin. Kinetin did not enhance germination. The pattern of germination over time in culture varied with cytokinin type. After 4 and 8 weeks, a higher percentage of the final germination had occurred on media with BA and 2iP. Nine protocorm morphological types were identified. The morphological types observed with the use of kinetin were similar to those seen with the control, whereas on BA and 2iP the morphological types were different. Protocorms developed faster on all three cytokinins than on the control. Benzyl adenine added at the highest concentrations delayed root development. A model to explain the effect of cytokinins on protocorm development is proposed.

Introduction

Cypripedium candidum Muhl. ex Willd., commonly called the small white lady's-slipper, is a north temperate terrestrial orchid. *Cypripedium candidum* is commonly found in moist sites of tallgrass prairie and is considered to be in danger of extinction throughout most of its range in North America (Brownell 1981). It currently occurs in 2 Canadian provinces, with Manitoba having the largest number of stands, and in 13

states in the U.S. (Bowles 1983). The major factor in its decline is habitat destruction primarily by human activity (Brownell 1981). To prevent its possible extinction, a reliable propagation method is desirable. One such method is using *in vitro* seed germination to produce plants for reestablishment in the wild or commercial production.

In general, *in vitro* germination of north temperate terrestrial orchid species seed has been more difficult than with tropical epiphytic orchids. Terrestrial species have more stringent requirements for germination but little is known about specific their requirements (Fast 1982). Nevertheless, in recent years certain requirements have become apparent. Terrestrial orchid species prefer a salt-poor medium and the nitrogen source plays a critical role in germination (Fast 1982; Van Waes and Debergh 1986b). Moreover, the required culture conditions are specific to genera and sometimes even to species (Henrich et al. 1981).

Experiments with plant hormones produced inconsistent germination results and these differences may again be related to genera (Arditti 1979; Arditti and Ernst 1984). Although specific hormones are included in many germination media, some of these hormones have little effect on germination and are only required for the developing protocorm (Harvais 1982), leading to confusion regarding their exact role.

Terrestrial orchid species are dependent on mycorrhizal associations during the later stages of germination in nature. The embryo may acquire the necessary elements for germination, including hormones (specifically cytokinins, Arditti 1979) or enzyme precursors (Hadley and Pegg 1989) from the associated endophyte. This may explain the possible need for cytokinins during asymbiotic germination. Harvais (1982) reported that

cytokinins are the most important growth regulators affecting *in vitro* germination of native terrestrial orchids. A cytokinin requirement has been shown for the germination of *C. reginae* (Harvais 1982) and *C. calceolus* L. (Van Waes and Debergh 1986b). However, little is known about the *in vitro* germination requirements of *C. candidum*, including the possible role of cytokinins. As well, germination rates are usually low in this species (De Pauw and Remphrey 1993; Henrich et al. 1981; Stoutamire 1990; Anderson 1990). The following investigation examines the cytokinin preference and optimum cytokinin levels for the *in vitro* germination and protocorm growth of *C. candidum*.

Materials and Methods

Pollination and Pod Collection. A large number of flowers of *Cypripedium candidum* in a stand near Kleefeld, Manitoba were manually self-pollinated, using the wide end of a flat toothpick to transfer both pollinia onto the stigma of the same flower (Harvais 1980). Six pods were randomly collected 8 weeks after pollination which had been determined in a previous study to be optimum for germination, although some variation in this optimum time exists (De Pauw and Remphrey 1993). Pods were brought to the lab and prepared for inoculation onto media on the same day.

Inoculation. In a laminar flowhood, whole pods were sterilized in a 2% sodium hypochlorite solution for 15 minutes, and then rinsed 3 times in sterile deionized water.

Pods were cut open and the seeds sprinkled onto a sterile piece of Whatman No. 2 filter paper that had been placed on top of the culture medium just prior to inoculation. The filter paper had been soaked overnight in 70% ethanol and then rinsed in distilled water prior to use. Seeds from each individual pod were assigned to all treatments. There were approximately 150 seeds per plate and 2 replications of each treatment per pod. Petri dishes were placed in an incubator at 23°C, in constant darkness. Seeds were subcultured at 6-week intervals, by simply transferring the filter paper onto fresh medium.

Culture Medium. The basic medium used was Norstog (1973) as modified by De Pauw and Remphrey (1993). The medium was supplemented with one of the following cytokinins: BA, kinetin and 2iP at concentrations of 0.1, 0.2, 0.4, 0.8 and 1.6 mg L^{-1}, and the control plates had no cytokinin.

Germination. Each plate was examined at 4-week intervals for 20 weeks in culture using a Zeiss stereomicroscope (10X magnification). The number of germinating seeds was recorded. Germination was considered to have occurred when the embryo emerged from the testa. Germination was expressed as a percentage of the total number of seeds inoculated.

Protocorm Development. The germinated embryo is called a protocorm (Arditti 1967). Protocorm morphology was assessed in relation to cytokinin type and concentration at

12 and 20 weeks of culture by classifying them into one of 9 morphological types encompassing growth from germination to rooting (see Results section). Representative morphological types were photographed using Wild M8 dissecting microscope equipped with a Photoautomat camera attachment. The frequency of protocorms of each morphological type was calculated as a percentage of the total.

Statistical Analysis. Analysis of variance (ANOVA) and tests for the least significant difference (LSD) were performed on germination percentages for each 4-week interval. The model included partitioning of variance for pod, cytokinin type, cytokinin concentration and replications. Row by column contingency tests using Chi-square were performed to investigate differences in protocorm development between treatments.

Results

Germination

The effect of three different cytokinins and increasing cytokinin concentrations on the germination of *C. candidum* after 20 weeks in culture are shown in Fig. 2.1. Both BA and 2iP increased germination significantly compared to the control with no cytokinin, whereas germination in the presence of kinetin was not significantly different from the control, regardless of concentration. For both 2iP and BA, germination increased with concentration until 0.8 mg L^{-1}. Statistical tests performed on germination data at each 4-week interval (Appendix C), revealed that the relative differences among cytokinin types and cytokinin concentrations found after 20 weeks were the same after 4 weeks and throughout the entire time in culture.

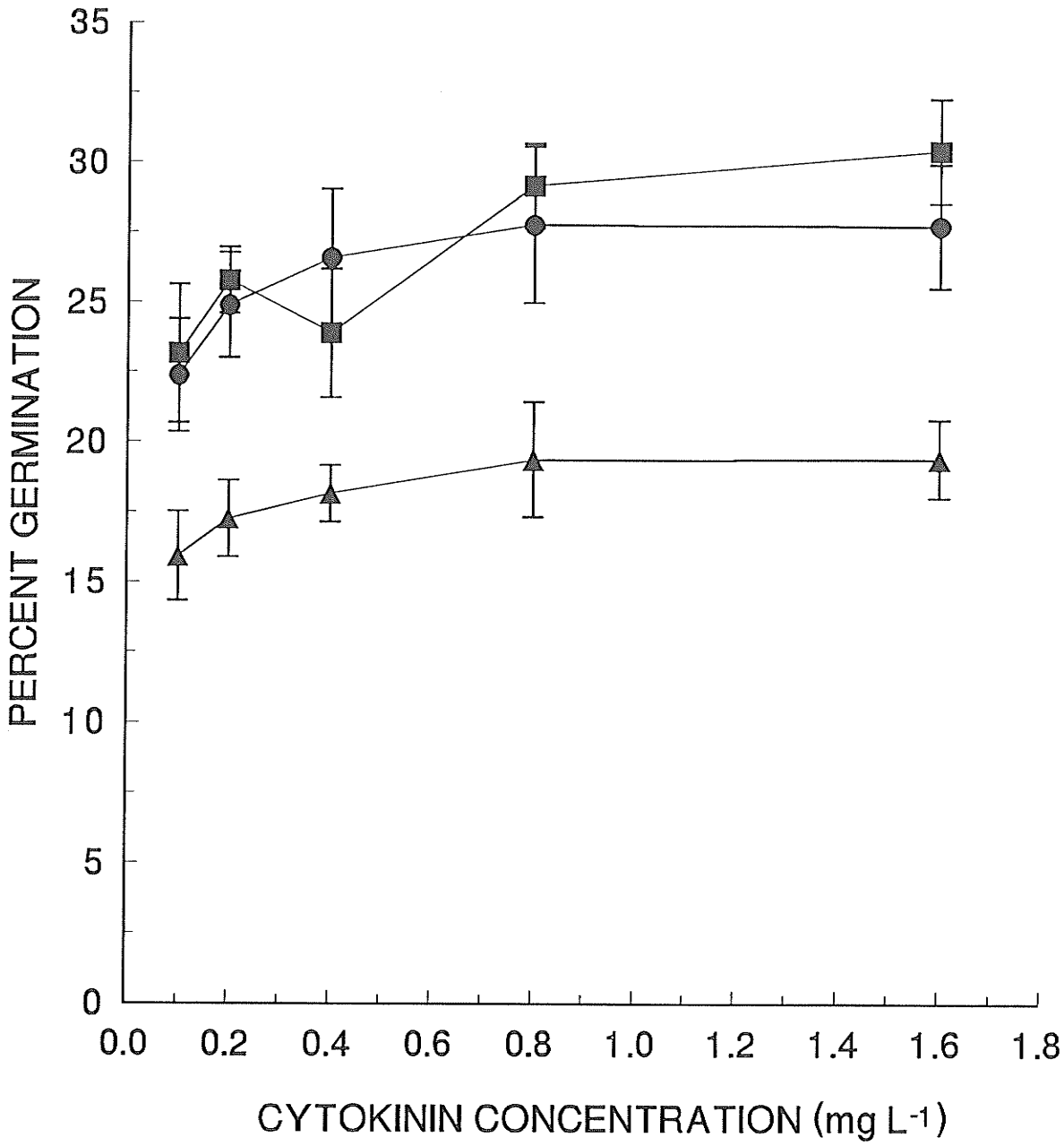


FIG. 2.1. The effect of three cytokinins at various concentrations on the germination of *C. candidum* seed after 20 weeks in culture, showing mean percent germination ± standard error. Kinetin (▲); BA (●); 2iP (■). The mean percent germination of the control with no cytokinin = 16.94 ± 1.39. From ANOVA, significance levels were: cytokinin, $p<0.0001$, concentration, $p<0.0001$, cytokinin x concentration interaction, $p=0.7076$.

There were differences in the pattern of germination among types of cytokinin used over the time in culture. Germination was initiated approximately 2 weeks after inoculation in most treatments. After 4 and 8 weeks in culture, a higher percentage of the final germination had occurred on media with cytokinins, but particularly on those using 2iP and BA (Table 2.1). However, after 12 weeks in culture further increases in germination were small regardless of cytokinin type. Approximately 90% of the total number germinated by 20 weeks had germinated after twelve weeks in culture, regardless of cytokinin type used.

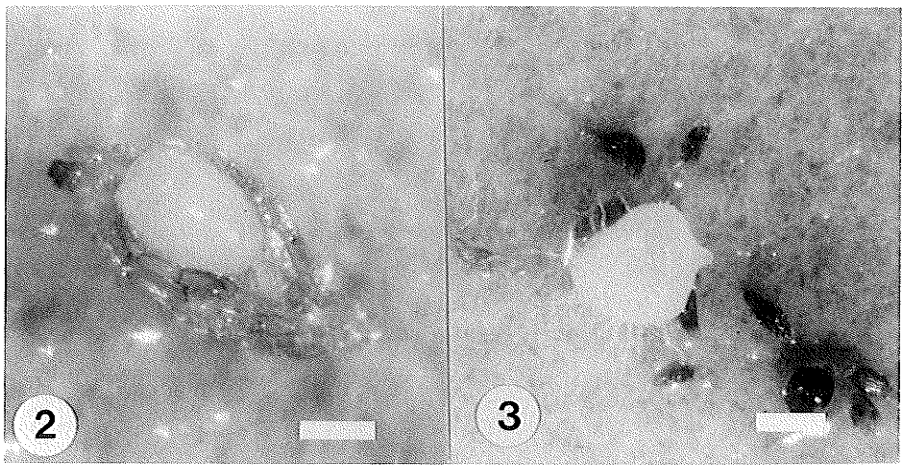
Protocorm Morphology and Development

Nine morphological protocorm types were distinguished over the time in culture (20 weeks). The first distinct protocorm structure was recognized when the protocorm had just broken through the testa forming a small spherical mass (Stage 1, Fig. 2.2). A second group of protocorms was recognized in which the protocorms were about 2 times larger than those in Stage 1 and less rounded, sometimes with a distinctive point at one end, and the presence or absence of rhizoids on the other end (Stage 2, Fig. 2.3). These two stages were recognized in all cultures and represented the initial stages of germination. Such stages are reportedly common to most orchid species (Arditti 1967).

Table 2.1. Relative germination of *C. candidum* seed after 4, 8, 12 and 16 weeks in culture expressed as a percentage of the total number of seeds that had germinated after 20 weeks. For each cytokinin, values were pooled across concentration. BA, Benzyl adenine; 2iP, 6-(α,α-dimethylallylamino)-purine.

Treatment	Weeks in culture			
	4	8	12	16
Control	34.38	57.90	89.96	96.20
Kinetin	39.09	66.33	89.99	94.44
2iP	54.48	81.11	90.91	94.27
BA	47.70	75.89	90.05	94.54

FIGS. 2.2 and 2.3. Initial germination stages of *C. candidum*. FIG. 2.2. Stage 1 protocorm. Bar equals 0.2 mm. FIG. 2.3. Stage 2 protocorm. Bar equals 1.0 mm.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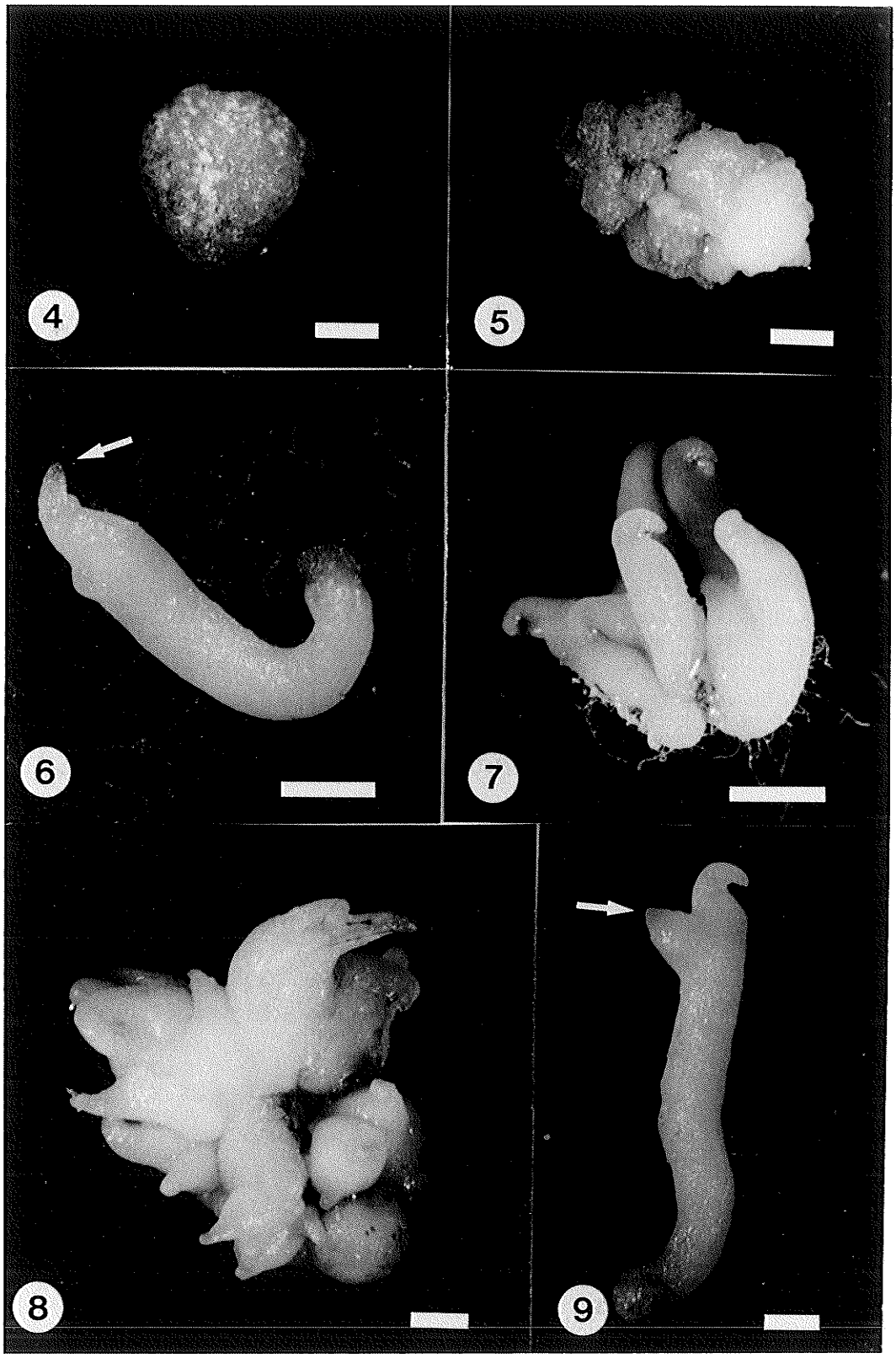
Following the initial stages of germination, several protocorm morphological types were recognized on the plates prior to the formation of roots: (i) large and amorphous protocorm bodies 2 to 3 times the size of those in Stage 2. There were two sub-types, amorphous protocorm bodies having an uneven surface (APB; Fig. 2.4) and amorphous protocorm bodies with obvious protrusions or bumps on the surface (APB-P; Fig. 2.5), (ii) elongated single protocorms with distinctively hooked meristems and root hairs usually present on the middle and bottom portion (SPB; Fig. 2.6), and (iii) two types of multiple protocorm bodies (MPB). MPB-1 appeared like a collection of elongated protocorms (similar to SPB) originating from one protocorm (Fig. 2.7). MPB-2 were particularly large but individual protocorms were not distinct as in MPB-1 (Fig. 2.8). Protocorms that turned brown were designated as Br.

As roots began to develop, two stages were recognized: those with roots just beginning to form (RPB; Fig. 2.9) and those with one or several long roots, now considered as seedlings (Sdl; Figs. 2.10 and 2.11). Distinctions between the morphological types were no longer made once roots were formed.

There were differences in protocorm morphology associated with the type and concentration of cytokinin. Regardless of morphological type, protocorms on control plates and on kinetin were consistently smaller than on the other cytokinins. Without cytokinins, the most common protocorm morphological types were APBs, SPBs and MPB-1s if RPBs and Sdl are not considered (Fig. 2.12a-c). With the addition of kinetin, especially at lower concentrations, protocorm morphology closely resembled that observed without cytokinins (Fig. 2.12a). With the addition of BA (Fig. 2.12b) or

FIGS. 2.4 to 2.9. Protocorm morphological types of *C. candidum*. FIG. 2.4. Amorphous protocorm body (APB). FIG. 2.5. Amorphous protocorm body with protrusions (APB-P). FIG. 2.6. Single protocorm body (SPB). Arrow indicates shoot meristem. FIG. 2.7. Multiple protocorm body, type 1 (MPB-1). FIG. 2.8. Multiple protocorm body, type 2 (MPB-2). FIG. 2.9. Rooting protocorm body (RPB). Arrow indicates root initial. Bar equals 1.0 mm. See results section for description of each type.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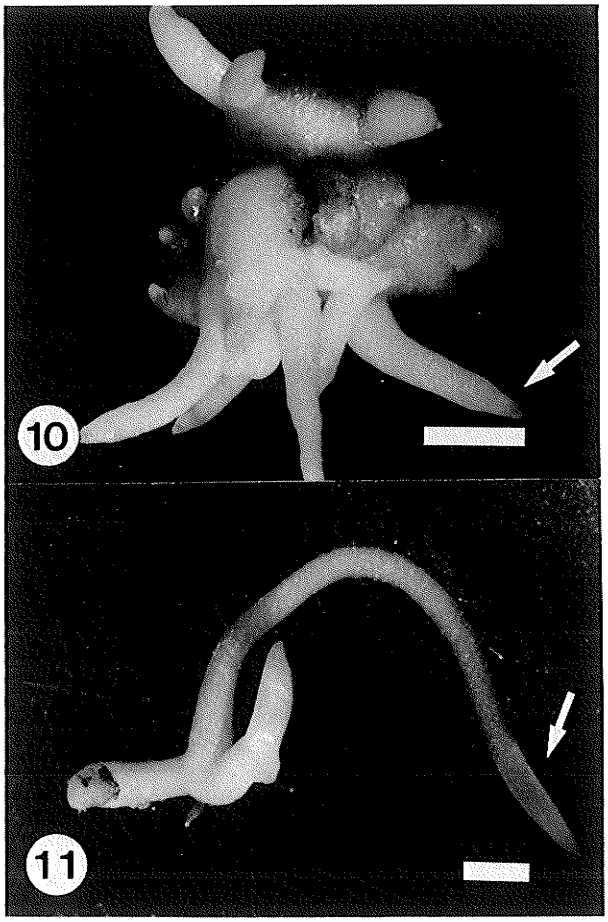
FIGS. 2.10 and 2.11. Seedlings (Sdl) of *C. candidum* showing roots (arrows). Bar equals 2 mm.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2iP (Fig. 2.12c) to the germination medium, protocorm morphology deviated from that observed with the control or kinetin. SPBs were rarely seen, especially at higher concentrations. MPB-1s did not occur. Instead, the morphological types APB-Ps, especially in the case of 2iP, and MPB-2s were most common.

Rooting occurred on single and multiple protocorms and, particularly in the case of 2iP, on APB-Ps. The root formation response was related to hormone type and concentration (Fig. 2.12a-c). On low concentrations of BA, there was a higher percentage of Sdls compared to the control. Increasing concentrations resulted in fewer Sdls. In contrast, on kinetin and 2iP, the percentage of Sdls generally increased with concentration and, in the case of kinetin, there is a corresponding decrease in SPBs, suggesting that the SPBs have formed roots.

The frequencies of each morphological type varied with time in culture in relation to cytokinin type. After 12 weeks in culture, protocorm development was slower in the control than with all cytokinins tested (Fig. 2.13a). Without cytokinin, over 50% of the protocorms had not developed beyond Stage 2. None had reached the rooting stages. With the addition of cytokinins, a larger proportion of protocorms developed beyond Stage 2 and this effect was especially evident on 2iP and BA compared to kinetin. Nevertheless, on each cytokinin approximately equal percentages of protocorms were at the seedling stage.

After twenty weeks in culture some germination was still occurring in all treatments (Stage 1, Fig. 2.13b). The proportion of protocorms that had formed roots was greater than at 12 weeks. This was particularly evident for protocorms in the presence of cytokinins.

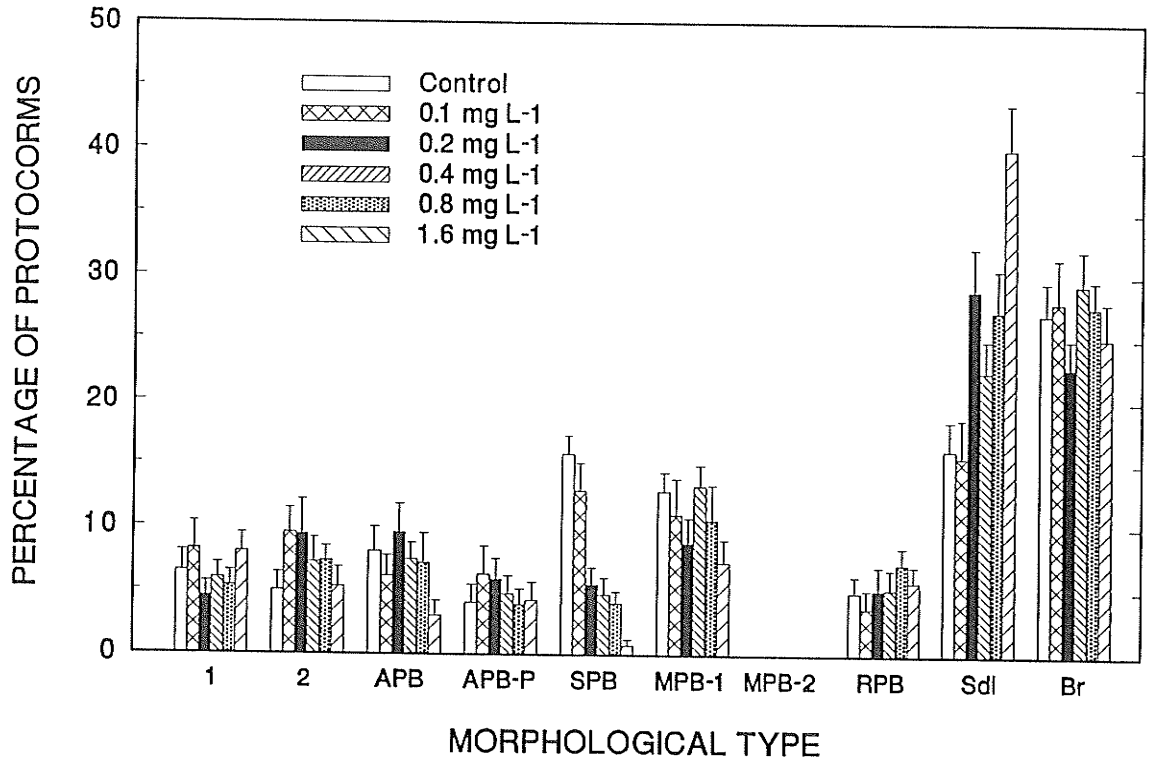

FIG. 2.12a. Frequency of each protocorm morphological type of *C. candidum* on kinetin calculated as a percentage of the total number of protocorms after 20 weeks in culture at six concentrations, including a control. Row x column contingency test of independence using χ^2 statistics (df=15) for Sdl protocorms: cytokinin concentration x cytokinin type, $\chi^2 = 1495$; $p<0.0001$.

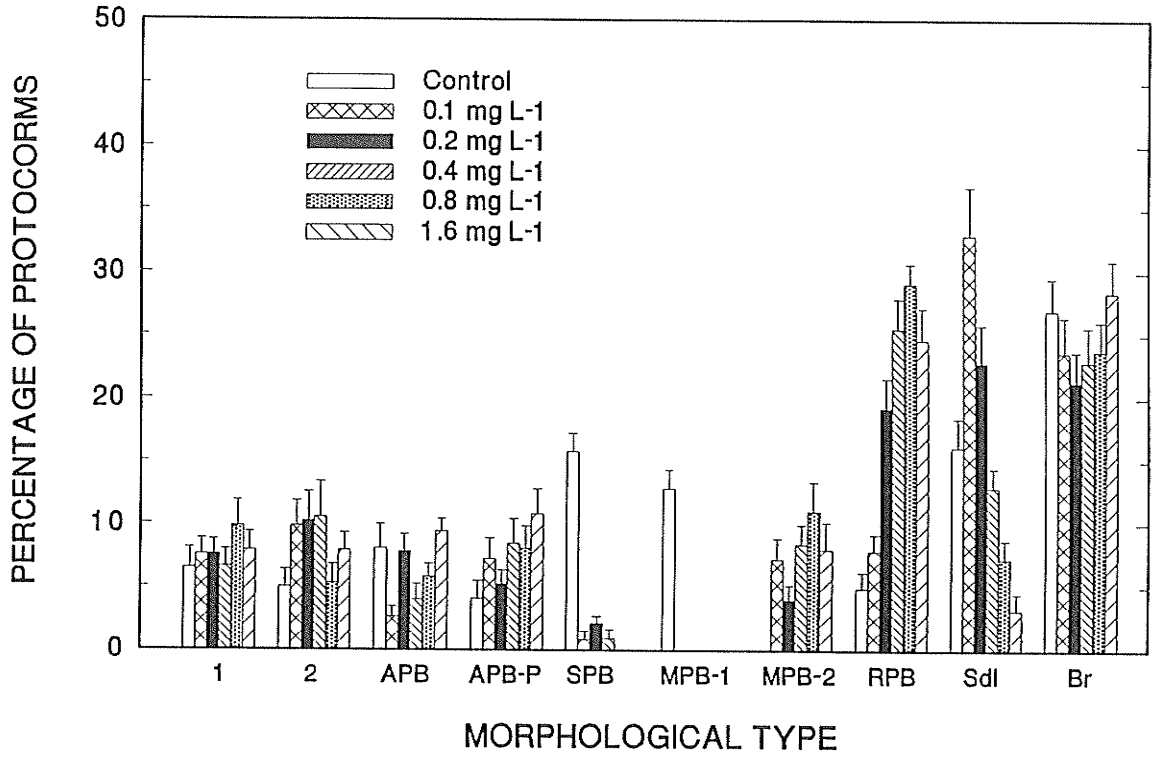

FIG. 2.12b. Frequency of each protocorm morphological type of *C. candidum* on BA calculated as a percentage of the total number of protocorms after 20 weeks in culture at six concentrations, including a control. Row x column contingency test of independence using χ^2 statistics (df=15) for Sdl protocorms: cytokinin concentration x cytokinin type, $\chi^2 = 1495$; $p<0.0001$.

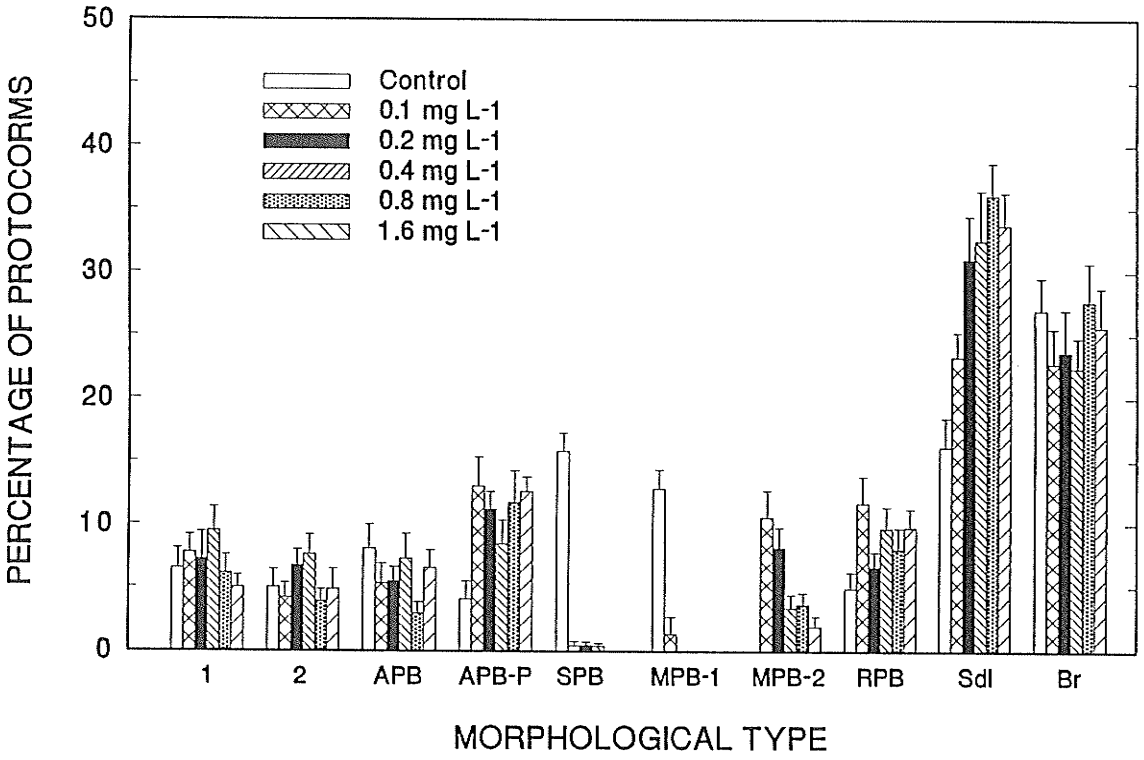

FIG. 2.12c. Frequency of each protocorm morphological type of *C. candidum* on 2iP calculated as a percentage of the total number of protocorms after 20 weeks in culture at six concentrations, including a control. Row x column contingency test of independence using χ^2 statistics (df=15) for SdI protocorms: cytokinin concentration x cytokinin type, $\chi^2 = 1495$; $p<0.0001$.

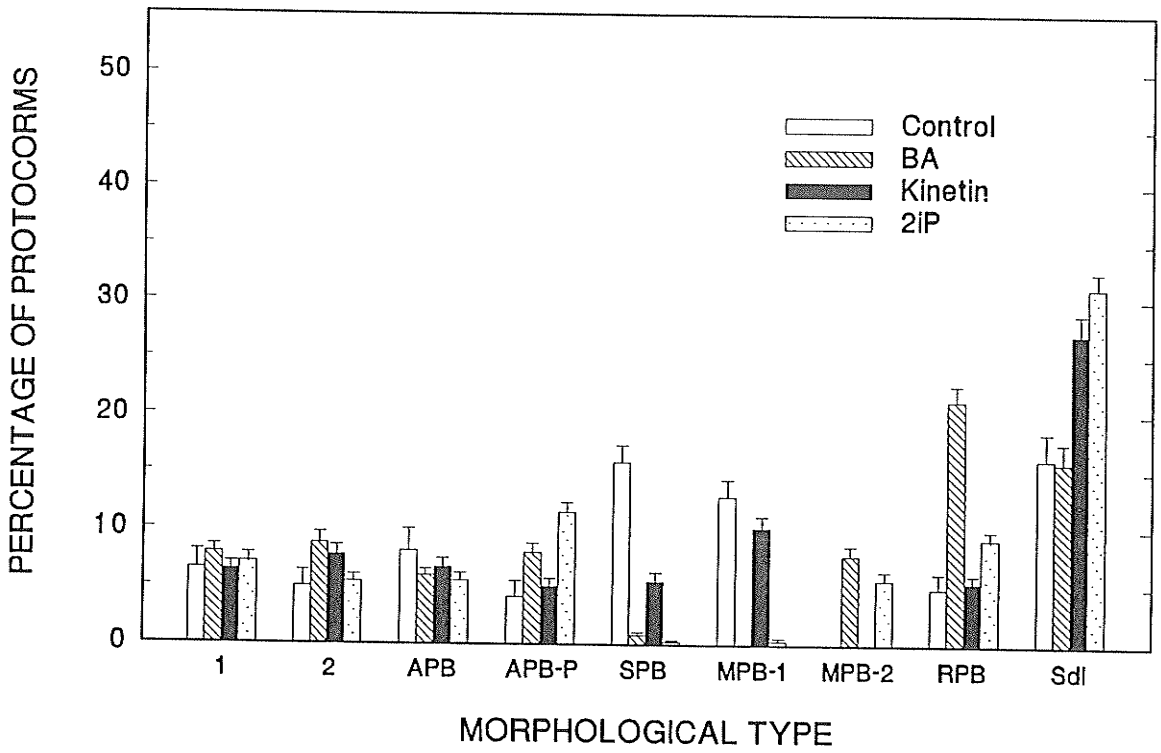

FIG. 2.13a. Frequency of each protocorm morphological type of *C. candidum* after twelve weeks in culture calculated as a percentage of the total number of protocorms on each cytokinin type. Values are means and standard error pooled across concentration for each cytokinin. Row x column contingency tests of independence using χ^2 statistics (df=7): time in culture x morphological stage for kinetin, $\chi^2 = 266$, $p=0.0001$; BA, $\chi^2 = 300$, $p<0.0001$; 2iP, $\chi^2 = 338$, $p<0.0001$; control, $\chi^2 = 109$, $p<0.0001$.

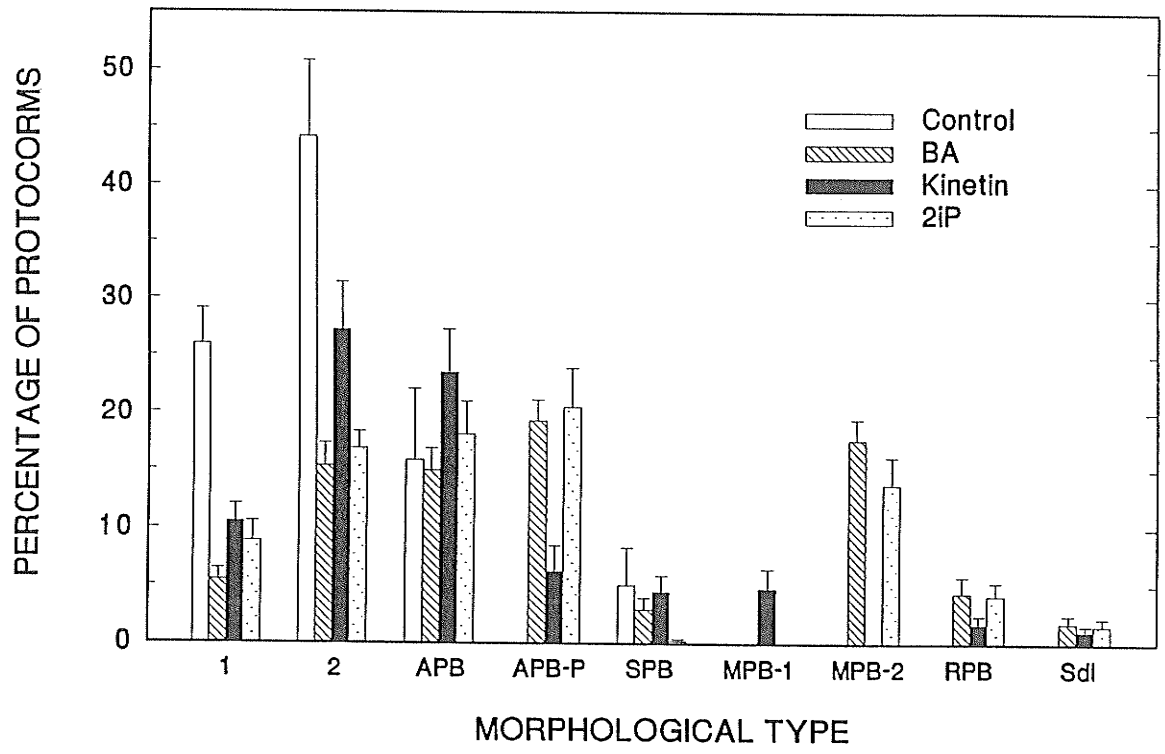

FIG. 2.13b. Frequency of each protocorm morphological type of *C. candidum* after twenty weeks in culture calculated as a percentage of the total number of protocorms on each cytokinin type. Values are means and standard error pooled across concentration for each cytokinin. Row x column contingency tests of independence using χ^2 statistics (df=7): time in culture x morphological stage for kinetin, $\chi^2 = 266$, $p=0.0001$; BA, $\chi^2 = 300$, $p<0.0001$; 2iP, $\chi^2 = 338$, $p<0.0001$; control, $\chi^2 = 109$, $p<0.0001$.

Browning and subsequent death of protocorms was common and occurred most frequently at the initial stages of germination when the protocorm was at Stage 1. However, browning was not restricted to Stage 1 (data not shown) and there was no particular trend in relation to the type of cytokinin and concentration used (Br, Fig. 2.12a-c).

Discussion

In general, orchid seeds respond to cytokinins either by an increase or a decrease in germination or there is no effect (Arditti and Ernst 1984). The particular response depends on the species. *Cypripedium* species appear to require a cytokinin for germination and this has been demonstrated for *C. reginae* (Harvais 1982) and *C. calceolus* (Van Waes and Debergh 1986b). In the time frame studied, some germination of *C. candidum* occurred without the addition of cytokinins to the medium but in the presence of two of the three cytokinins tested, germination clearly improved. Addition of BA and 2iP enhanced germination whereas kinetin had little effect. For BA and 2iP, germination generally increased with increasing concentrations to an apparent maximum at 0.8 mg L^{-1}. Higher concentrations did not increase germination dramatically. Harvais (1982) found a similar response in *C. reginae*. Low concentrations of 2iP and BA were more effective than kinetin at high concentrations in stimulating germination. Such research helps to explain some of the inconsistencies regarding the germination response of orchid seeds to cytokinins. For example, *C. candidum* obviously has a cytokinin

preference and if kinetin had been tested in isolation, a different conclusion would have been reached regarding the effectiveness of cytokinins in enhancing germination. Similarly, Blonstein et al. (1991) observed that the inhibition of seed germination of *Nicotiana plumbaginifolia* also showed differential response to cytokinins.

In *C. candidum*, some germination occurred without the addition of cytokinins to the media. Perhaps endogenous cytokinins are present but at inadequate levels in those seeds that did not germinate. Orchid species that do not require an exogenous cytokinin source for germination are cytokinin autonomous because they have high endogenous levels, an example being *Epidendrum fulgens* (Mercier and Kerbauy 1991).

The exact function of cytokinins in germination is unknown but there is evidence that in seeds with high levels of storage lipids, such as pecan nuts, cytokinins may play an important role in lipid mobilization (Dimalla and Van Staden 1977). Orchid seeds have no endosperm and no cotyledons and lipid droplets in the embryo are the primary storage material (Arditti 1979; Arditti and Ernst 1984). The requirement for cytokinins in the germination medium may thus be related to lipid utilization. It has been shown that if storage lipids cannot be utilized, germination will not continue (Manning and Van Staden 1987).

Lower germination of *C. candidum* was recorded in the present study than was achieved in previous studies (De Pauw and Remphrey 1993) for seed collected eight weeks after pollination. One explanation may be that the optimum time for collecting seeds was surpassed. Seed development can be affected by environmental conditions and so the optimum time to collect pods for maximum germination may vary from year to

year (Light 1989). Another explanation may be high variability in germination potential from pod to pod (Harvais 1980; De Pauw and Remphrey 1993). Another factor involved in the lower germination response may be the decline of reproductive ability. The cost of seed production to the plant can be high, resulting in decline of plant vigor, particularly after four consecutive years (Primack and Hall 1990). Since all flowers were manually pollinated to ensure seed set and the same site was used as a seed source for four consecutive years, plant vigor may be decreasing and consequently seed viability may also be reduced.

Based on the protocorm morphologies observed, a general hypothesis regarding their developmental sequence in response to the hormone treatments is proposed (Fig. 2.14). As mentioned, Stages 1 and 2 are sequential and occur in all treatments. After Stage 2, development diverges leading to the formation of either multiple or single protocorm bodies. SPBs form when Stage 2 protocorms elongate, and then eventually form roots (RPB and Sdl). This pathway occurs primarily on the control and kinetin plates. In the formation of multiple protocorms, a different pathway occurs in that Stage 2 protocorms develop to APBs and then to APB-Ps. The latter then form either MPB-1s or MPB-2s. The cytokinin type seems to affect the kind of multiple protocorm produced. On the control and kinetin, MPB-1s are the most common type whereas BA and 2iP usually lead to the formation of MPB-2s. An alternate pathway on the control and kinetin plates is that SPBs proliferate to form MPB-1s. Both multiple protocorm types advance to the rooting stages. In some cases, particularly on 2iP, APB-Ps advance directly to the rooting stages.

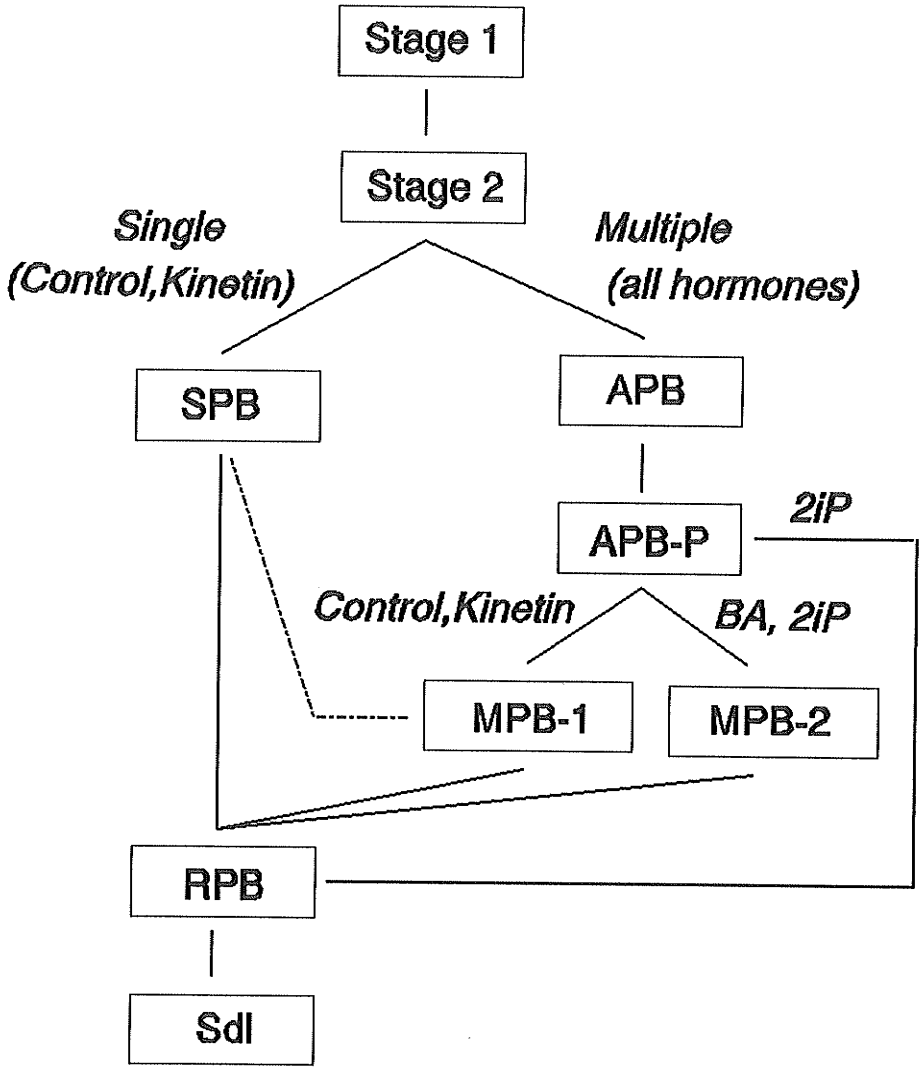

FIG. 2.14. Proposed model for the protocorm development of *C. candidum* in relation to cytokinin type. Abbreviations for each morphological type are as follows: APB, amorphous protocorm body; APB-P, amorphous protocorm body with protrusions; SPB, single protocorm body; MPB-1, multiple protocorm body, type 1; MPB-2, multiple protocorm body, type 2; RPB, rooting protocorm body; Sdl, seedling.

The observation that MPB-1s form on control plates suggests that the formation of multiple protocorms is not primarily a response to exogenous cytokinins. Nevertheless, if the proposed model is considered, the addition of cytokinins, particularly BA and 2iP resulted in a higher proportion of multiple protocorms, seemingly at the expense of single protocorm formation. Development on kinetin followed similar pathways as the control except that at higher concentrations, there was an increased proportion of protocorms that formed roots. The decrease in the proportion of SPB with increasing kinetin concentration is related to the fact that the protocorms have advanced to the rooting stages. Protocorm development was particularly sensitive to both BA and 2iP, even at low concentrations. The production of MPB-1 did not occur and multiple protocorms with a different morphology were produced (MPB-2, Fig. 2.14). Moreover, the highest concentrations of BA delayed root growth, as seen by the high proportion of protocorms just beginning to form roots (RPBs) and fewer Sdls (Fig. 2.12b), but the reverse was true at lower concentrations. An inhibitory effect of BA on root elongation has been noted in other orchid species (Colli and Kerbauy 1993).

The differential growth response of protocorms to each cytokinin is a common phenomenon that has been observed in most plant tissues (Bosse and Van Staden 1989; Van der Krieken et al. 1990). Harvais (1982) also found differences in protocorm growth of *C. reginae* in response to cytokinins, and kinetin and BA in that order, were the most suitable compared with 2iP. The reasons for differential growth responses to cytokinins is not completely understood but may be due to differences in cytokinin metabolism (Horgan 1984; Mok et al. 1985). Van der Krieken et al. (1990) found that

the differential response of tobacco explants to cytokinins was not due to differences in uptake from the medium. Instead, they suggest that cytokinins bind to one receptor which has a differential sensitivity to the hormones.

Browning or necrosis and subsequent death of the protocorm is a common phenomenon in terrestrial orchids (Stoutamire 1974; Harvais 1982). Stoutamire (1974) suggested that death of protocorms may be due to inappropriate culture conditions, such as improperly balanced nutrients or the lack of required growth-stimulating substances. The present study suggests that cytokinins neither prevent nor increase death of protocorms. Death of protocorms may simply be a characteristic of the seed population. It is possible that inviable embryos can imbibe enough water to expand and break through the testa but not develop further. However, approximately 25% of protocorms were lost as the result of browning, thereby reducing the final germination, and further work is required to reduce this loss.

Research on *in vitro* germination of native orchid seeds is often a long, slow process due to the considerable periods of time required for germination to occur. From this and a previous study (De Pauw and Remphrey 1993), it is apparent that a high percentage of seeds germinate within three months of culture. After this point, the increase in germination is very small. Also, differences in germination between treatments are evident after just one month. Therefore, it is reasonable to conclude that treatment differences can be accurately assessed at this time, although the extent of the differences will not be apparent until 3 months of culture. It is hoped that the results of this investigation may facilitate future research into the specific germination requirements of *Cypripedium* species.

In conclusion, *Cypripedium candidum* has a definite cytokinin preference for germination and protocorm development. Kinetin is less effective for germination but results in more normal protocorm development and is required at relatively high concentrations. On the other hand, 2iP and BA enhance germination significantly at concentrations less than 1.0 mg L^{-1}. Small amounts cause marked changes in protocorm development and increase the production of multiple protocorms (MPB-2). Benzyl adenine at low levels (0.1 mg L^{-1}) is more effective than 2iP for protocorm growth and may be useful for the development of a method of meristem culture for vegetative propagation similar to tropical species (Morel 1974). The proposed model for protocorm development is based on morphologies observed at two discrete time periods. Further work is required to verify the model by following the development of individual protocorms throughout the entire time in culture.

GENERAL DISCUSSION AND CONCLUSIONS

It is generally accepted that *in vitro* seed germination of *Cypripedium* species is more difficult than for other terrestrial orchids (Linden 1992). Germination has been demonstrated many times but the results have been confusing and contradictory. Germination as low or lower than 1% has been reported, with slow or arrested protocorm development (Arditti et al. 1984; Henrich et al. 1981). The research presented in this thesis shows that *in vitro* germination of three *Cypripedium* species is not only possible but has the potential to be a viable method of propagation. Of the three species tested, *C. reginae* appeared to be the most amenable to culture, with high germination and vigorous protocorm growth. *C. calceolus* var. *parviflorum* and *C. candidum* also responded favourably, with slightly lower but adequate germination and good protocorm growth. This has special significance in the case of *C. candidum* because of its endangered status in Canada, including the Province of Manitoba. Research on *C. candidum* has been very limited and again, germination has been reported to be very poor and slow, taking up to six months to occur, and with protocorms often dying (Stoutamire 1990; Anderson 1990). Using the methods described in this thesis, germination and protocorm growth of *C. candidum* was significantly improved. However, approximately 25% of the protocorms did turn brown and die. This would suggest that, although germination conditions have been improved, the optimum requirements have not yet been met. Further work is needed to reduce these losses.

Germination of mature seed is often poor and the use of immature seed from green pods has been shown to improve germination. My data supports these observations and demonstrates that germination of immature seed is superior to that of mature seed. More importantly, an optimum time exists at which seeds should be collected to maximize germination. For all three species, germination peaked for seed collected eight weeks after pollination. As well, in the case of *C. candidum*, seed collected eight weeks after pollination had higher germination than the other times of collection. However, there is some variability in the optimum time of collection, as in the case of *C. candidum* in 1989 where germination peaked for seed collected 6 weeks after pollination instead of eight weeks, suggesting the possible importance of the developmental stage of the embryo. In all three species, germination decreased sharply after the optimum time of collection, as the seed approached maturity.

European-based studies on terrestrial orchid species often focus on enhancing germination of mature seed using various pretreatments. These studies are critical because most European terrestrial orchids are rare and seed is difficult to obtain. Thus, mature seed, which can be stored for several years, must be relied upon. In Canada, the use of mature seed is also important in those cases where species are rare or where plants are less accessible, making pollinating and collecting pods more difficult. However, in all other cases, the use of immature seed is the preferred method, for several reasons. First, for many orchid species, including certain *Cypripedium* species, accessibility is not a problem. Second, immature seed germinates successfully, without the requirement for a pretreatment. Moreover, pretreatments for mature seed would have to be optimized

for each individual species. Third, even if the pretreatment is optimized, mature seed may never reach the germination potential of immature seed because mature seed may be less viable. Finally, from a technical aspect, it is much easier to sterilize the whole green pod than to manipulate individual mature seeds through a number of pretreatments. The seed is extremely small in size and very hydrophobic which makes it difficult to handle. It is therefore concluded that the use of immature seed is the preferred and most practical choice and could be successfully and easily applied to propagation of *Cypripredium* species, either for commercial production or reestablishment in the wild.

Previous studies using Norstog medium for germination of *Cypripedium* species gave poor results (Henrich et al. 1981; Arditti et al. 1984). The modifications made to Norstog medium in the present study improved germination and were more favorable for germination and protocorm growth of all three species. Although modified Norstog was not the best medium for all species and at all times of collection, germination was as good as Van Waes and Debergh medium and, except in the case of *C. reginae*, was superior to Harvais medium. In the case of *C. candidum*, the rate of germination was greater with modified Norstog than the other two media tested. As well, protocorm development of all 3 species was better on modified Norstog compared to the other media tested. Protocorms had several long roots by the end of the experiments, which was not the case with the other two media.

Studies on the effect of hormones on germination of *Cypripedium* species is limited and somewhat misleading. Some literature suggests that both auxins and cytokinins are required for germination of *Cypripedium* species (Anderson 1990) but from the controlled

studies available, it appears that cytokinins alone are the critical hormones (Harvais 1982; Van Waes and Debergh 1986b). The results of the present study show that cytokinins are an important requirement for germination of *C. candidum*. However, a cytokinin preference was demonstrated for germination and protocorm growth. Benzyl adenine and 2iP increased germination significantly compared to a control without cytokinins. On the other hand, kinetin had no effect. This differential response to cytokinins may explain the inconsistent results of other studies with respect to the importance of cytokinins in affecting germination. Protocorm growth following germination also showed a differential response to cytokinins. Growth of protocorms on kinetin closely resembled that seen on the control and seems to represent a more "normal" type of development. With BA and 2iP the morphological types observed were different and sometimes anomalous. Protocorms developed faster on all three cytokinins compared to the control.

The research presented in this thesis has provided answers to many basic questions regarding the germination of three *Cypripedium* species, including the best time to collect seed to maximize germination and an appropriate medium for germination and protocorm growth. This provides a basis for further studies that could quickly provide information on the germination requirements of *Cypripedium* or other terrestrial orchid species. For example, modified Norstog medium is completely defined which makes it particularly suited to investigate other factors that affect germination, such as the optimum nitrogen source (including organic versus inorganic nitrogen sources) and the effects of different concentrations of macroelements. These are factors that have been shown to affect germination and growth in other terrestrial species.

The use of filter paper for the present germination studies proved to be a successful technique. A filter paper was placed on the medium and seeds were sprinkled on top. Due to the minute size of the seed, it is impossible to transfer them individually. In most studies, the seeds are kept on the same medium for 6 months or more. During this time, desiccation of the medium occurs and phenolic compounds accumulate. By transferring the whole filter paper, seeds are easily moved to fresh medium to avoid these problems, without interfering with water or nutrient uptake. This technique is very useful and could facilitate future studies. For example, as the study on the effect of cytokinins on *C. candidum* suggests, the requirement for cytokinins changes from germination to the protocorm stages. In the case of BA, high levels (0.8 mg L^{-1}) are necessary for maximum germination, but lower levels (0.1 mg L^{-1}) are required for protocorm growth and root formation. Similarly, kinetin seems inappropriate for germination but suitable for normal protocorm growth. Therefore, a medium containing BA could be used for germination and then cultures could be transferred to a medium with kinetin or with diminishing concentrations of BA for further protocorm development. Filter paper would facilitate transferring of cultures as the needs of the developing protocorms change.

More research is required to verify the proposed hypothesis of the effect of cytokinins on protocorm development. By following individual protocorms throughout the culture period one would be provided with more information on the differential affect that cytokinins have on protocorm growth. This information could be used to manipulate protocorms in order to optimize growth or to develop a vegetative propagation method.

For example, BA causes proliferation of the protocorm and this characteristic could be used to develop a vegetative propagation method. Kinetin, on the other hand seems appropriate for direct production of plantlets. The development of a vegetative propagation method for a threatened species using tissue culture is difficult. Meristems are often the tissue source of choice but this requires destruction of the plant. In contrast, seed propagation is a non destructive method and seeds are produced in great abundance. Germinated protocorms could be a useful tissue source for developing vegetative methods. In this way, plants from the wild would not have to be destroyed for research material.

In terms of protocorm growth, a standardized system for describing development is required. The observations of the present study provide the beginnings of such a system. This type of system has been used by Hailes and Seaton (1989) and Spoerl (1948) to compare growth of *Cattleya* on different media. In this technique, sequential stages of protocorm growth are recognized to provide a standard growth pattern. Then the number of protocorms at each stage is multiplied by the Stage Number. These values are summed to give a Protocorm Growth Index (PGI). Without a standard method such as the one described, it is very difficult to describe differences in development between treatments or to compare results from different studies. However, a standard growth index would be required for individual species because, even within the *Cypripedium* genus, the patterns of protocorm growth are different.

If the methods described in this thesis are to be useful for propagation, further work is required on tranplanting the *in vitro* germinated seedlings from the petri dish to

soil. Literature is available on the cultivation of mature plants (Holman 1976; Muick 1978; Olver 1981) and a few studies have successively grown seedlings from tissue culture in pots (Bailes et al. 1987; Anderson 1990; Frosch 1986). However, specific information is unavailable or unknown. For example, a cold treatment is required to get leaf extension but the optimum conditions are not known. Other questions are: At what stage should the seedlings be transferred from the petri dish into soil? What type of potting medium is required? Is the pH of the potting medium important? In their natural habitat *Cypripedium* species take 10 to 16 years from germination to first flower and the first aerial leaf is not seen until the third year following germination. This time period must be decreased if propagation methods are to become viable. Although transplanting seedlings was not part of the present investigation, the seedlings obtained from the germination studies were transplanted into soil, with some success. From this work, it would appear that tissue culture has decreased the time to the first aerial leaf by approximately two years. This stage was achieved in one year, instead of three.

Orchid species in general are among the most endangered plant species due to habitat destruction, overcollection and illegal smuggling. Commercial propagation via tissue culture techniques can play an important role in saving these species by eliminating over-collection from the wild and thereby reducing the pressure on natural populations. In Canada, species such as *C. reginae* and *C. calceolus* may not be endangered at this time but propagation would eliminate the common practice of removing them from the wild. Propagation would also allow reintroduction of endangered species, such as *C. candidum*, to their native habitats. Reintroduction accompanied by careful management

(Sheviak 1990) could successfully repopulate a species. In this light, seed propagation is the preferred method, as opposed to mass vegetative propagation, in order to maintain genetic diversity. Reintroduction of orchid species is currently being investigated in a few locations (Mitchell 1990; Rubluo et al., pers. comm.). The *Cypripedium* species are beautiful native plants that deserve our attention in order to preserve them in their natural habitats and to prevent the decline which has occurred in other parts of the world.

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APPENDICES

APPENDIX A: Media Used for Germination Studies of *Cypripedium* Species

	Harvais	Van Waes and Debergh	Modified Norstog
Macroelements (mg L^{-1})			
NH_4NO_3	1400		
KH_2PO_4	200	299.20	455
$MgSO_4.7H_2O$	200	100.86	370
KNO_3	200		
KCl	100		375
$Ca(NO_3)_2.4H_2O$	400		
$CaCl_2.2H_2O$			370
KI	0.10		
ammonium citrate	19.0		
Fe-EDTA		43.0	
Fe-citrate			10
Microelements (mg L^{-1})			
$MnSO_4.4H_2O$	2.03	25.00	3.00
H_3BO_3	0.50	10.00	0.50
$ZnSO_4.7H_2O$	0.50	10.00	0.50
$Na_2MoO_4.2H_2O$	0.02	0.25	0.025
$CuSO_4.5H_2O$	0.50	0.025	0.025
$CoCl_2.6H_2O$			0.025
Vitamins (mg L^{-1})			
thiamine HCl	5.0	0.50	
niacin	10.0	5.00	
calcium pantothenate	5.0		
myo-inositol		990.0	
pyridoxine HCl		0.50	

folic acid		0.50	
biotin		0.05	
Complex Additives			
potato extract (%)	5.0		
casein hydrolysate (mg L^{-1})		500.0	
Amino Acids (mg L^{-1})			
L-glutamine		102.31	400
L-alanine			50
L-cysteine			20
L-arginine			10
L-leucine			10
L-phenylalanine			10
L-tyrosine			10
Hormones (mg L^{-1})			
kinetin	1.00		
NAA	0.10		
BA		0.2	0.2
Carbohydrate (g L^{-1})			
dextrose	0.02		
sucrose		19.85	34.2
Miscellaneous			
malic acid			1.0
agar			6.0

APPENDIX B: Analysis of variance (ANOVA) tables.

Table B-1. Separate ANOVAs for each time of seed collection (weeks after pollination) for mean percent germination of *C. reginae* among media.

Source of variation	Degrees of freedom	6 WAP		8 WAP		10 WAP		12 WAP	
		F	P	F	P	F	P	F	P
Pod	3	2.67	0.221	12.45	0.005	30.79	0.001	17.67	0.009
Media	2	1.87	0.297	0.80	0.4939	0.99	0.435	2.58	0.190

Table B-2. Separate ANOVAs for each time of seed collection for mean percent germination of *C. calceolus* var. *parviflorum* among media.

Source of variation	Degrees of freedom	6 WAP		8 WAP		10 WAP		12 WAP	
		F	P	F	P	F	P	F	P
Pod	3	3.65	0.225	5.44	0.068	2.37	0.248	13.67	0.004
Media	2	4.05	0.198	1.10	0.417	25.54	0.013	8.93	0.016

Table B-3. Separate ANOVAs for each time of seed collection (WAP) for mean percent germination of *C. candidum* in 1989 among media.

Source of variation	Degrees of freedom	6 WAP		8 WAP		10 WAP		12 WAP	
		F	P	F	P	F	P	F	P
Pod	3	12.84[1]	0.039	4.17	0.065	n/a[2]	n/a	0.24	0.863
Media	2	21.33	0.017	2.80	0.138	n/a	n/a	18.30	0.003

[1]Degrees of freedom=2.
[2]ANOVA could not be performed because of missing values due to contamination.

Table B-4. Separate ANOVAs for each date of seed collection (WAP) for mean percent germination of *C. candidum* in 1990 among media.

Source of variation	Degrees of freedom	5 WAP		6 WAP		7 WAP		8 WAP		10 WAP		12 WAP	
		F	P	F	P	F	P	F	P	F	P	F	P
Pod	1	2.69	0.108	4.49	0.035	13.76	0.003	22.26	0.002	0.10	0.767	4.76	0.061
Media	2	3.31	0.072	4.87	0.028	9.43	0.008	10.22	0.006	9.44	0.008	15.41	0.002

APPENDIX C: Two-way ANOVA table of percent germination of *C. candidum* among cytokinin type and cytokinin concentration for successive weeks in culture (refer to Chapter 2).

Source of variation	Degrees of freedom	F	P
4 weeks in culture			
Pod	5	9.36	<0.0001
Cytokinin type	2	48.88	<0.0001
Pod*Cytokinin type	15	2.67	0.0036
Cytokinin concentration	4	7.52	<0.0001
Type*Concentration	8	0.53	0.8325
8 weeks in culture			
Pod	5	9.54	<0.0001
Cytokinin type	2	70.75	<0.0001
Pod*Cytokinin type	15	3.27	0.0005
Cytokinin concentration	4	14.01	<0.0001
Type*Concentration	8	0.86	0.5513
12 weeks in culture			
Pod	5	12.20	<0.0001
Cytokinin type	2	41.14	<0.0001
Pod*Cytokinin type	15	2.24	0.0141
Cytokinin concentration	4	10.16	<0.0001
Type*Concentration	8	1.06	0.4047
16 weeks in culture			
Pod	5	12.68	<0.0001
Cytokinin type	2	49.30	<0.0001
Pod*Cytokinin type	15	2.01	0.0296
Cytokinin concentration	4	10.44	<0.0001
Type*Concentration	8	1.22	0.3037
20 weeks in culture			
Pod	5	11.90	<0.0001
Cytokinin Type	2	52.01	<0.0001
Pod*Cytokinin Type	15	2.17	0.0179
Cytokinin Concentration	4	7.79	<0.0001
Type*Concentration	8	0.68	0.7076

APPENDIX D - Preliminary tests using the modified tetrazolium chloride method to assess seed viability in three *Cypripedium* species.

Introduction

Many of the standard seed viability tests are unsuccessful with native terrestrial orchids (Harvais 1982). However, Van Waes and Debergh (1986a;1986b) developed modifications to the tetrazolium chloride (TTC) method to test the viability of mature seed of 31 Western European terrestrial orchids, including one *Cypripedium* species. The modifications involved pretreating the seeds in calcium hypochlorite (a standard solution used for sterilization of tissue prior to culture) before applying the standard TTC test. The required length of the pretreatment was dependent on the species and appears to be directly related to the impermeability of the seed coat (see Literature Review). However, this test has not been applied to seed at different stages of maturity. Therefore, in 1989, preliminary tests were performed to assess suitability of the modified tetrazolium chloride method for measuring the viability of seed at different stages of maturity in *C. candidum*, *C. calceolus* var. *parviflorum* and *C. reginae*.

Materials and Methods

Pods of *C. candidum*, *C. calceolus* var. *parviflorum* and *C. reginae* were collected at 6, 8, 10 and 12 weeks after pollination (WAP). Four pods were collected at each time of collection (refer to Materials and Methods, Chap. 1). Seed from individual pods that was not used for germination experiments was retained for the TTC tests.

A 1% tetrazolium chloride solution was prepared in the following manner: 10 g of 2, 3, 5-triphenyltetrazolium chloride was dissolved in 1 l of distilled water. The pH was adjusted to 7 with 10 N NaOH. The solution was stored in darkness at room temperature.

The modified TTC test involved pretreatment of seeds in 5% sodium hypochlorite ($Na(COl)_2$) + Tween-20 solution, rinse in sterile distilled water, prior to application of the standard TTC test. For the standard TTC test, seeds were placed in small bottles with 10 ml of a 1% TTC solution. The bottles were sealed and placed in darkness at 30°C for 24 hours. After 24 hours, the solution was removed and seeds were washed 3 times in sterile distilled water. Viable embryos turn pink and nonviable embryos remain white.

Both the modified and the standard TTC tests were applied to most seed. In the modified TTC test, the optimal pretreatment times for seed at different stages of maturity were unknown, therefore they were estimated based on the color of the seed coat. After application of the TTC test, seeds were observed under a dissecting microscope and embryos were classified as pink, slightly pink, white or shriveled and empty. Only those embryos that were completely pink or red were considered as potentially viable (Van Waes and Debergh 1986a).

Results

In all 3 species, seed collected at 6 WAP was white and, in the case of *C. calceolus* var. *parviflorum* and *C. candidum*, the seed was very moist. When the seeds

were removed from the pod, pieces of the placenta came with the seeds. After the standard TTC treatment, embryos of *C. calceolus* var. *parviflorum* and *C. candidum* showed no staining. However, the placenta attached to the seeds turned pink. For *C. calceolus* var. *parviflorum* a pretreatment was performed for seed collected 6 WAP, but no staining occurred on the embryos or the placenta, suggesting that the $Ca(OCl)_2$ harmed the tissue. For this reason, pretreatment was not performed on the other 2 species for seed collected 6 and 8 WAP. For *C. reginae*, embryos from seed collected 6 WAP did react to the stain without a pretreatment (Table D-1). In three of the four pods, the percent pink embryos was higher than the percent germination.

As the seed matured, the seed coat turned brown and the seeds were loose. At 8 WAP, there was a mixture of brown and white seeds in the same pod. For all 3 species, seed collected 8 WAP showed good reaction to the stain, without a pretreatment (Table D-1, D-2, D-3). However, in *C. calceolus* var. *parviflorum* and *C. candidum* the percent germination was lower than the percent pink embryos in most cases. In *C. reginae*, the percent germination was higher than the percent pink embryos.

By 10 and 12 WAP, seed was brown, dry and hydrophobic in all species. Without the pretreatment, seed collected at 12 WAP floated on top of the tetrazolium, resulting in poor staining reaction. After the pretreatment, seed was less hydrophobic and the percent pink embryos increased compared with no pretreatment. However, the reaction to the pretreatment varied between species and between individual pods and, in some cases, the increase in percent pink embryos with pretreatment was very small or there was even a slight decrease. There appeared to be no particular correlation between the percent pink embryos, with or without pretreatment, and the percent germination.

Table D-1. Percent germination and percent pink embryos after modified TTC test for individual pods of *Cypripedium reginae*.

Pod	Weeks After Pollination	Percent Germination[1]	% Pink Embryos	
			No Pretreatment	With Pretreatment[2]
CR-1	6	45.06	78.08	N/A[3]
CR-2	6	48.42	79.07	N/A
CR-3	6	46.03	65.00	N/A
CR-4	6	67.82	54.42	N/A
CR-5	8	92.57	38.78	N/A
CR-6	8	58.33	19.83	N/A
CR-7	8	23.02	15.45	N/A
CR-8	8	84.00	49.08	N/A
CR-9	10	78.50	9.02	21.74
CR-10	10	68.75	23.35	50.00
CR-11	10	3.99	0.50	3.81
CR-12	10	3.95	0.50	4.66
CR-13	12	0.34	0.00	3.13
CR-14	12	0.00	0.00	1.00
CR-15	12	12.25	0.97	0.85
CR-16	12	9.30	0.00	2.54

[1]Percent germination after 20 weeks in culture on the best medium.

[2]10 min pretreatment in 5% sodium hypochlorite + Tween-20, 30 min rinse in distilled water.

[3]Not applicable. A pretreatment was not performed on seed collected at 6 and 8 WAP (see Results section).

Table D-2. The percent germination and percent pink embryos after modified TTC test for individual pods of *Cypripedium candidum*.

Pod	Weeks After Pollination	Percent Germination[1]	Percent Pink Embryos	
			No Pretreatment	With Pretreatment[2]
Ca-4	8	28.34	53.05	N/A[3]
Ca-5	8	14.41	63.64	N/A
Ca-6	8	22.43	46.15	N/A
Ca-7	8	26.74	44.98	N/A
Ca-10	10	6.56	19.11	15.03
Ca-11	10	9.09	11.56	10.88
Ca-12	12	22.06	0.00	0.87
Ca-13	12	19.02	0.00	0.47
Ca-14	12	29.66	0.00	21.35
Ca-15	12	21.76	3.68	13.82

[1]Percent germination after 20 weeks in culture on the best medium.

[2]For seed collected 10 WAP, pretreatment was 10-15 minutes in 5% sodium hypochlorite + Tween-20. A 30 minute pretreatment was performed on seed collected 12 WAP.

[3]Not applicable. A pretreatment was not performed on seed collected 8 WAP (see Results section).

Table D-3. Percent germination and percent pink embryos after modified TTC test for individual pods of *Cypripedium calceolus* var. *parviflorum*.

Pod	Weeks After Pollination	Percent Germination[1]	Percent Pink Embryos	
			No Pretreatment	With Pretreatment[2]
Cs-11	8	52.78	58.00	0.00
Cs-12	8	53.85	4.76	0.00
Cs-23	8	35.15	61.82	0.00
Cs-25	8	30.03	80.28	0.00
Cs-28	10	2.99	60.26	29.44
Cs-29	10	14.58	13.07	35.66
Cs-30	10	10.10	11.63	30.41
Cs-31	10	1.47	27.59	41.78
Cs-37	12	11.25	1.25	1.93
Cs-41	12	2.98	1.25	3.04
Cs-42	12	3.80	0.00	0.00
Cs-48	12	9.06	0.00	0.00

[1]Percent germination after 20 weeks in culture on the best medium.

[2]15 min pretreatment in 5% sodium hypochlorite + Tween-20 for seed collected 8 and 10 WAP, 30 min pretreatment for seed collected 12 WAP.

Discussion

Van Waes and Debergh (1986a, 1986b) showed that pretreatment in calcium hypochlorite was necessary to get optimal uptake of tetrazolium in mature seeds. For *C. calceolus* the optimal pretreatment was 4 hours. The hypochlorite solution acted as an oxidant to remove suberin in the integuments and thus increased permeability to the stain. The darker integuments contained more suberin, and therefore, longer pretreatment times were required. In my preliminary studies, seed collected at 10 and 12 WAP was brown in color and, especially at 12 WAP, was very hydrophobic and impermeable, resulting in a poor staining reaction. Pretreatment increased uptake of the stain, resulting in more pink embryos. However, the pretreatment may not have been optimal because there is a poor correlation between the percent pink embryos and the percent germination.

In contrast, seed collected at 6 WAP was still white, suggesting that the deposition of suberins in the seed coat had not yet occurred. Therefore, a pretreatment was not necessary. For seed collected 8 WAP, uptake of the tetrazolium solution did not appear to be significantly impaired so a pretreatment was not necessary. In some cases, the percent pink embryos was higher than the percent germination. This could suggest that the germination conditions were inadequate and that the germination potential was not reached. However, in the case of *C. reginae*, the percent pink embryos was less than the percent germination, suggesting that uptake of the stain was impaired. It should be pointed out that, in general, viability tests are not 100% accurate, and should be viewed with some caution (Mayer and Poljakoff-Mayber 1989).

Another factor to consider is the use of sodium hypochlorite in the present study instead of calcium hypochlorite, as was used by Van Waes and Debergh (1986a). Van Waes and Debergh (1986b) recommended a pretreatment time for *C. calceolus* of 4 hours, when using calcium hypochlorite. Sodium hypochlorite is a much stronger sterilant and therefore, the required pretreatment times would be much shorter. It was necessary to estimate the appropriate time for pretreatment so they may not have been optimal, resulting in poor correlation between the percent pink embryos and the percent germination.

In conclusion, there appears to be poor correlation between the percent pink embryos and the percent germination, with or without a pretreatment, for all three species at all times of seed collection. The seed coat characteristics changed at each time of collection. Also, there was variability among species in the seed coat characteristics for seed collected at the same time. Therefore the pretreatment times may not have been optimal and further work would be required to optimize the pretreatment for each species and for each time of seed collection.

The research presented in this thesis suggests that 8 WAP is the best time to collect seed in order to optimize germination. Therefore, further work should concentrate on optimizing viability tests for that time of seed collection. From these preliminary tests, it appears that seed collected at 8 WAP may not require a pretreatment or a very short pretreatment.

Viability tests are an important tool when studying germination. If conditions could be optimized for testing the seed viability of *Cypripedium* species, it would help to increase our knowledge on the germination, or lack of germination of these species.